Effects of salinity, atrazine, molinate and chlorpyrifos individually and as mixtures to the freshwater alga *Pseudokirchneriella subcapitata* and cladoceran *Daphnia carinata*

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Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and preparation of the thesis itself has been acknowledged. In addition, I certify that all information and literature used are indicated in the thesis.

Signature of Candidate

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Neil Hemantha Dassanayake

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Thesis structure

The experimental chapters in this thesis are written as journal articles. Since journal articles must be self-contained, there is some degree of repetition in the thesis.

Chapter 1 discusses the importance of multigenerational studies in toxicity evaluations, the limitations of single species toxicity testing, and need for population and community level studies. Selection of suitable test organisms i.e., *Pseudokirchneriella subcapitata* and *Daphnia carinata* is also discussed. In addition, a review of inland salinity issues, pesticide pollution and importance of mixture toxicity studies including salinity and pesticide mixtures are discussed. Chapters 2 and 5 examine the exposure of *P. subcapitata* and *D. carinata* to salinity over several generations (separately) and life history and population level effects are discussed.

Chapters 3 and 6 describe the toxicities of salinity, atrazine, molinate and chlorpyrifos individually, and mixtures of different combinations of the toxicants to *P. subcapitata* (short-term chronic tests) and *D. carinata* (acute tests).

Chapters 4 and 7 examine the effects of high salinity acclimation (over multiple generations) of *P. subcapitata* and *D. carinata* on the toxicities of salinity, atrazine, molinate and chlorpyrifos individually and as mixtures of different combinations to determine whether there are sensitivity changes.

Chapter 8 summarises the findings of the study and evaluates the comparative effects of salinity (multigenerational and short-term toxicity), and three pesticides individually and mixtures on the two test organisms studied. The possible indirect effects of toxicants on organisms in higher trophic levels, i.e., *D. carinata* as a result of direct effects on the primary producers i.e., *P. subcapitata* are discussed. The importance of incorporating mixture toxicities and community level effects into environmental management of toxicants are discussed in relation to results of the present study.

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Abstract

The salinisation of inland water bodies occurs in many parts of the world. This issue is serious in Australia, especially in agricultural areas where pollution caused by pesticides is also prevalent. Even though mixture toxicity studies have been carried out for various classes of chemicals, little information is available on the toxicities of the combined effects of pesticides and salinity on freshwater aquatic biota. Aquatic organisms inhabiting salt-affected inland water bodies experience the effects of salinity over generations. Thus, there may be differences in the sensitivity of these organisms to salt-induced changes from those caused by other toxicants. There is no scientific information on how salinity-affected freshwater organisms respond to various agrochemicals in such environments.

Two representative test species, viz. the freshwater alga *Pseudokirchneriella subcapitata* and the freshwater cladoceran *Daphnia carinata* were selected to study the effects of salinity individually and as mixtures with selected pesticides (atrazine, molinate and chlorpyrifos).

The experiments consisted of effects of multigenerational exposure to salinity on the life history traits of these two species. The acute toxicities of individual toxicants and their mixtures on these two species were also studied. Finally, the acute toxicities of individual and mixtures of toxicants on both species acclimatised to salinity over multiple generations were compared with non-acclimatised populations. *P. subcapitata* (normal salinity 100 µS/cm) exposed to elevated salinities (3000 and 6000 µS/cm) over five sequential cultures (C0 – C4) had significant ($p \leq 0.05$) adverse effects on growth rate, number of cell divisions per day and generation time except for the second culture (C1). All cultures from C1 to C4 had significantly lower ($p \leq 0.05$) cell yields than C0 for both the elevated salinities. Significant differences ($p \leq 0.05$) between cultures were observed (compared with C0) in terms of the growth rate, cell divisions per day, generation time and cell yield indicating that stress induced by elevated salinity persisted over the five sequential cultures.

D. carinata (normal salinity 200 µS/cm) were maintained at elevated salinities (2000, 4000, 5500 and 6300 µS/cm) for four generations (F0 - F3). Salinities $\geq$ 5500 µS/cm

significantly reduced ($p \leq 0.05$) their reproductive ability, intrinsic rate of natural increase and ingestion rates. Neonatal length was significantly reduced ($p \leq 0.05$) at $\geq$ 2000 µS/cm while filtration rate was significantly reduced ($p \leq 0.05$) at $\geq$ 5500 and 4000 µS/cm for the F1 and F2 generations, respectively; no significant differences were found for the F0 generation. Significant delays ($p \leq 0.05$) in the reproduction also occurred with increasing salinities.Development of tolerance to salinity as an adaptation over generations was not clearly evident from these results. Cladoceran populations may be impacted due to reduced food availability (due to direct effects of salinity on the alga) at salinities $\geq$ 3000 µS/cm. Such cascading effects on species at higher trophic levels could result in community level effects. Thus, reliance on results of single species experiments is questionable and there should be a mechanism to incorporate community level toxic effects in ecological risk assessments and water quality guidelines.

Chronic toxicities of individual toxicants (72-hour IC50 values) to *P. subcapitata* were 5600 µS/cm, 48 µg/L, 300 µg/L, 797 µg/L for salt, atrazine, molinate and chlorpyrifos, respectively. Atrazine was very highly toxic, molinate was highly toxic and chlorpyrifos was moderately toxic to the alga. Toxicities of mixtures of these toxicants were evaluated using the Toxic Unit (TU) approach. Approximately 50% of the mixture combinations for non-acclimatised *P. subcapitata* conformed to antagonism, 47.3% to additivity and 1.8% to synergism. For salinity acclimatised and non-acclimatised cultures of *P. subcapitata,* chlorpyrifos/salt and atrazine/chlorpyrifos/salt mixtures were significantly ($p \leq 0.05$) different and high salinity acclimatised cultures were less tolerant of these mixtures. In salinity acclimatised cultures exposed to mixtures of the pesticides and salinity, the majority of the concentrations (71%) tested conformed to additivity. Approximately 20% and 9% of the mixtures conformed to antagonism and synergism, respectively. Changes in toxicity relationships in mixtures occurred between non-acclimatised and acclimatised cultures i.e., antagonistic relationships in non-acclimatised cultures became additive in acclimatised cultures, and some additive relationships in the non-acclimatised cultures became synergistic in the acclimatised cultures. This indicates an increase in the sensitivity to the toxicant mixtures of the acclimatised alga.

For *D. carinata,* acute toxicity (48-hour immobilisation EC50) values were 8790 µS/cm, 42 mg/L, 25 mg/L and 0.21 µg/L for salinity, atrazine, molinate and chlorpyrifos, respectively. Chlorpyrifos was very highly toxic while atrazine and molinate were slightly toxic to the cladoceran. Toxicity relationships for mixtures of pesticides and salinity for non-acclimatised cultures were 47% conformed to additivity, 11% conformed to synergism and 42% conformed to antagonism. The 11% of mixtures showing synergistic relationships needs attention in developing water quality guidelines (WQGs).

Acclimatisation to 6300 µS/cm water did not change the tolerance of *D. carinata* to acute exposures of atrazine, chlorpyrifos or molinate individually, as mixtures of the pesticides or as mixtures of the pesticides with the high salinity tested. Thus, acclimatisation to salinity did not adversely alter their defence mechanisms against individual pesticides or their mixtures. The toxicity relationships of mixture combinations were antagonism - 37%, additivity - 52% and synergism - about 11%. This suggests greater toxicity than expected from the concentration addition (CA) model. There was about a 5% decrease in antagonism in high salinity cultures compared with normal salinity cultures while additivity increased by the same percentage in high salinity cultures; synergism remained the same in both cultures.

The two test species exhibited different toxicity responses to the mixtures of toxicants. There were changes in sensitivities in terms of toxicity relationships in salinity-acclimatised and non-acclimatised cultures especially for *P. subcapitata.* The most prevalant toxicity relationship was additivity and therefore incorporation of additivity into the derivation or implementation of WQGs would protect the two species from approximately 90% of the mixtures tested. However, mixtures that have synergistic relationships, which were about 10% of the mixtures would not be sufficiently addressed by these WQGs. Based on the findings of the present study, it is recommended that long-term exposure scenarios and toxicities of mixtures should be incorporated to provide a more conservative approach in risk assessments and in deriving water quality guidelines.

Chapter 1

Rationale for the Thesis

1.1. Introduction

The increase of salinity in inland aquatic systems is considered as a highly significant environmental problem in certain parts of the world. Australia has suffered from inland salinity over time (Williams 1987; Williams 1999; Jolly, Williamson et al. 2001). Salinisation could be due to natural processes; however, human-induced factors have contributed to the rapid increase of salinity and it is an ongoing process at present (Jolly et al. 2001).

Point sources of pollution of inland waters are generally industrial effluents, sewage and municipality wastes, mining effluents etc., for which the sources and points of discharge can be identified. Non-point source pollution is more diffuse, and includes aerial deposits and surface runoff over large areas. One major non-point source pollutant is agricultural run-off, which contains pesticides and other agrochemicals. Point source pollution is relatively easy to monitor, and enforcement of regulations to control the level of input are relatively straightforward compared to non-point source pollutants. Non-point source pollutants can enter the waterways via surface water run-off and it is difficult to monitor them and quantify loads. Agrochemicals are currently the only group of highly toxic chemicals that are deliberately applied to the environment to control pests and the effects are widespread on receiving waters. Therefore, the use of pesticides in agriculture has attracted the concern of researchers and decision-makers as it is necessary to gain a better understanding of their consequences on affected environments and non-target organisms.

1.2. Inland salinity issues

1.2.1. Extent of inland salinity

Salinisation in inland areas of the world has been increasing over time (Jolly et al. 2001; Williams 1987). The extent and distribution of saline and sodic soils (soil which has the potential to develop salinity) in the world has been estimated by Szabolcs (1987) (Table 1.1).

Table 1.1: Worldwide distribution of saline and sodic soils. (Szabolcs 1987).

Region	Total Area (million Ha)	Saline and sodic soils (million Ha)	Saline and sodic soils (% of land area)
Africa	1,899.1	72.2	3.8
Asia, Pacific and Australia	3,107.2	443.7	14.3
Europe	2,010.8	79.4	3.9
Latin America	2,038.6	111.4	5.5
Near East	1,801.9	105.6	5.9
North America	1.923.7	19.1	1.0
Total	1.2781.3	831.4	6.5

About 6.5% of the world's land is affected by salinity which covers over 400 million hectares (Szabolcs 1994). Much of the world's land is not cultivated, but a significant proportion of cultivated land is salt-affected. Of the current 230 million ha of irrigated land, 45 million ha are salt-affected (19.5%) and of the 1,500 million ha under dryland agriculture, 32 million are salt-affected to varying degrees (2.1 percent) (Szabolcs 1994).

Inland salinity in Asia, the Pacific and Australia is considerably more extensive than the overall global situation (Table 1.1). The land area currently affected by salinity and the predicted increases over the next 50 years, if present trends continue, have been estimated in different states of Australia (Table 1.2) (National Land and Water Resource Audit, 2001). Percentage increases range from around 60% to over 90% in different states over this fifty-year period.

Table 1.2: The present extent of salt-affected lands in Australia and the estimated increases by the year 2050 based on the present trends in salinisation. (National Land and Water Resource Audit 2001).

State	Total land area (ha)	Salt-affected land area – 1998/2000 (ha)	Estimated salt-affected land area by 2050 (ha)	% increase
NSW	80,064,200	181,000	1,300,000	75.6
Victoria	22,741,600	670,000	3,110,000	64.6
Queensland	173,064,800	Not assessed	3,100,000	-
South Australia	98,348,200	390,000	600,000	87.8
Western Australia	252,987,500	4,363,000	8,800,000	90.6
Tasmania	6,840,100	54,000	90,000	88.7

1.2.2. Categories and sources of inland salinity

There are two broad categories of salinity in Australia: primary (natural) salinity and secondary (human-induced) salinity (DLWC 2000). The two main sources of natural salt deposits in Australia are cyclic and connate salts. Cyclic salts are salts that have evaporated from oceans with water and are then deposited in coastal areas (Herczeg et al. 2001). Rainwater generally contains 10 to 30 mg/l of salts (Lambert and Turner 2000). Salts from rainfall only become a problem when there is insufficient rain to flush the deposited salts from the soil profile, as is the case in Australia. The rain-deposited salt can be transported by wind and deposited in inland areas increasing salinity in such areas. Connate salt comes from sediments deposited by inland seas millions of years ago (Herczeg et al. 2001). For example, the Wiannamatta Shale Group of the Sydney Basin was deposited by a retreating ocean during the Triassic period (some 230 million years ago). These shales contain salt and are responsible for salinity problems in Western Sydney.

Human-induced salinisation is mainly caused by land-use practices. Irrigation is the major factor causing inland salinity in Australia and an example is the Murrumbidgee

Irrigation Area (MIA). Irrigation salinity occurs when the soil is waterlogged for irrigated crops, which causes the underground water table to rise and brings the salt already present in the subsurface to the surface. Removal of vegetation cover is another factor associated with salinisation. When deep-rooted plants are cleared, increased infiltration, reduced evapotranspiration, lack of rain water interception, retention capacity and increased run-off can occur (National Action Plan for Salinity and Water Quality 2000). All these factors facilitate transport of the sub-surface salt to the surface and thereby cause salinisation.

Other factors contributing to increased salinisation are the inherent properties of soil, that is, the salt content of the soils and porosity. High salt content is often found in certain soils which were subjected to salinisation while the area was under the sea. Soil porosity affects the potential of groundwater to rise through the soil profile. The greater the soil porosity, the greater the ability of groundwater to rise through the soil due to capillary action.

Geomorphological and geological features are also important in salinisation. Certain rocks with high salt content can lead to salinisation (Rengasamy 2006).

Climatic factors such as heavy rains can inundate and waterlog an area. Once the water has evaporated, the salt dissolved in the water remains in the soil surface. In certain situations the intrusion of seawater through the mouths of rivers and other waterways causes salinisation in low-lying areas (Woodroffe et al. 1993). In areas where heavy extraction of groundwater prevails, this can cause the shrinking of freshwater aquifers and the rise of salinisation (Nobi and Gupta 1997). The exact changes in salinity as a result of global climatic changes and sea level rise are not fully understood yet but there is certainly the potential for climate change to modify the current situation.

2.3. Features of Australian inland salinity

The ionic composition of the Australian freshwater systems differs from that of the world average, being more similar in composition to seawater (Bayly and Williams

1972). The world average composition of cations and anions in freshwater in decreasing order are $Ca^{2+}>Mg^{2+}>Na^{+}>K^{+}$ and $HCO_3^{-}>SO_4^{2-}>Cl^{-}$ (Buckney 1980). In certain salt-affected waters in Victoria, the order of the composition of ions are: $Na^{+}>Mg^{2+}>Ca^{2+}>K^{+}$ and $Cl^{-}>SO_4^{2-}>HCO_3^{-}>CO_3^{2-}$ (Halse et al. 1998), while the average seawater ionic composition is $Na^{+}>Mg^{2+}>C^{2+}a>K^{+}$ and $Cl^{-}>SO_4^{2-}>HCO_3^{-}>SO_3^{2-}$ (Wetzel 2001). In addition, inland salinity in Australia occurs due to the shallow and surface salt deposits and is therefore different from mine water, which is the principal source of salinity in some countries (Otero and Soler 2002).

Elevated salinity has been recorded in many parts of Australia including the Western Australian wheat belt (Pinder et al. 2005), and a number of river systems in NSW including the Murrumbidgee Irrigation Area (MIA) (National Land & Water Resources Audit 2001). The effects of salinity in these areas include changes in species sensitivity distributions (SSDs) (Kefford et al. 2006), and changes in fauna and flora .

Natural salinisation in Australia has been accelerated through human activities (Jolly et al. 2001). The accelerated rise in salinity in inland rivers and wetlands has occurred over 200 years due to land clearing and agricultural practices. Pinder et al. (2005) reported salinities in inland water bodies in the Western Australian wheat belt to range from <4300 to 140,000 µS/cm (Table 1.3), while Jolly et al. (2001) reported a mean increase of 4.37 µS/cm/year with a minimum of -6.9 to a maximum of 139.5 µS/cm/year in streams in the Murrumbidgee Irrigation Area (Table 1.4).

Table 1.3: Salinities of water bodies in Western Australian wheat belt (Pinder et al. 2005)

No. of sites	Salinity µS/cm
86	< 4,300
46	4,300 – 14,000
69	14,000 – 140,000

Table 1.4. Mean salinities and salinity trends in streams in Murrumbidgee Irrigation Area (Jolly et al. 2001)

	Mean ± Standard Error)	Minimum	Maximum
Salinity µS/cm	442 ± 81	46	6573
Salinity trend µS/cm/year	4.37 ± 2.03	-6.9	139.5

1.2.4. Effects of salinisation

Salinisation causes habitat degradation, creating stressful conditions and loss of biodiversity. A report on the implications of increased salinity concluded that it could cause the extinction of approximately 450 species of native flora and 250 species of invertebrate water fauna in the Western Australian wheat belt (Biodiversity Conservation & Management 2001). Another effect of salinisation is the potential loss of land suitable for agriculture and subsequent decline in production. For example, more than $130 million of agricultural production is lost annually through salinisation, about $9 million annually damage to roads and highways in southwest New South Wales (National Action Plan for Salinity and Water Quality 2000), and if not effectively managed within 20 years, the salt content in Adelaide's drinking water may exceed WHO standards for desirable drinking water (WHO 2006). More than $6 million are spent every year on building maintenance related to salinity in South Australia (National Action Plan for Salinity and Water Quality 2000). Even though the ecological effects of salinity were well-known, salinity is not considered as a toxicant in the Australian water quality guidelines rather it is considered as a physic-chemical parameter of water (ANZECC and ARMCANZ 2000).

1.3. Pesticide pollution

1.3.1. Pesticide use

The use of pesticides in agriculture is increasing rapidly in order to fulfil the demands of the world's rising population (Yudelman et al. 1988) (Table 1.5). A similar trend is observed in Australia (Australian Commodity Statistics 2007) (Figure 1.1).

Consequently, unless the toxicity of pesticides being used decreases over time, it can be assumed that the effects of pesticides on non-target organisms will increase.

Table 1.5: The world consumption of pesticides between years 1983 and 1998 (Yudelman et al. 1998)

World Pesticide Consumption, 1983-1998 Value (US$ millions)			
Region	1983	1993	1998
North America	3,991	7,377	8,980
Latin America	1,258	2,307	3,000
Western Europe	5,847	7,173	9,000
Eastern Europe	2,898	2,571	3,190
Africa/Mideast	942	1,258	1,610
Asia/Oceania	5,572	6,814	8,370
World Total	20,507	27,500	34,150

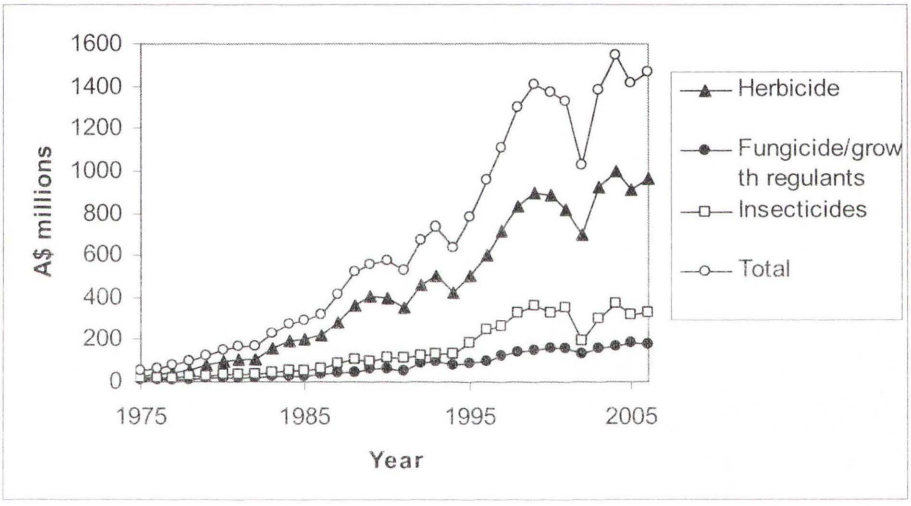

Figure 1.1: The trends in pesticide sales in Australia from 1975 to 2006 (Australian Commodity Statistics 2007).

1.3.2. Nature of pesticide pollution

The levels of pesticides in the aquatic environment depend on factors such as the spray period, weather conditions, application method, half-life of the pesticide, affinity to the sediments and bioavailability, etc. (Bowmer et al. 1998). The aquatic environment receives pesticides from farm areas via drift, run-off, direct overspray and leaching (Peterson et al. 1994) and these then undergo adsorption onto sediments and suspended matter, bioaccumulation and degradation, etc. depending on their physical and chemical characteristics and prevailing environmental conditions.

Persistent chemicals are now banned or have their use restricted for specific purposes (Chaudhry et al. 2002). The presence of pesticides in waterways of most agricultural regions is characterised by low concentrations for prolonged periods and high concentrations for relatively short periods. Therefore, unlike the salinity issue, it is important to understand both the short-term and long-term toxicities of pesticides on non-targeted organisms.

Understanding the effects of pesticides in the aquatic environment has been a challenge for researchers working in the field. The rationale for choosing the three test chemicals (i.e., atrazine, chlorpyrifos and molinate) is set out below. The key factors in their selection were use within Australia, mode of action, environmental fate and the amount of currently available toxicity data.

Atrazine, which inhibits the photosynthetic electron transport chain, is among the most heavily used herbicides in the world. It is the second most heavily used herbicide in Australia (Radcliffe 2002). The third most heavily used is the closely related herbicide, simazine (Radcliffe 2002). Approximately 2,550 kg of active ingredient of atrazine is used annually in the Murrumbidgee Irrigation Area (MIA) (Simpson and Haydon 1999). Concentrations as high as 100 µg/L have been recorded in waterways the MIA during the cropping season for rice and maize (Korth et al. 1995). Atrazine is relatively persistent and mobile in both surface and groundwater. It is found quite frequently in waterways including irrigation water, streams and groundwater in Australia (Bowmer et al. 1998). Effects of atrazine on a variety of non-targeted freshwater species have been fairly well studied e.g., on diatoms (Hoagland et al. 2002), shrimps, cladoceran, fish, and midges (Phyu et al. 2004; Phyu et al. 2005a; Phyu et al. 2005b; Phyu et al. 2006), and mussels (Bringolf et al. 2007).

Molinate inhibits cell division in plants. It is one of the most frequently applied herbicides in Australia (Bowmer et al. 1998; Radcliffe 2002). In addition, it is one of the more frequently found herbicides in intensive agricultural run-off and receiving waters in Australia and often exceeds the water quality guidelines, especially during the spray season between October and December (Bowmer et al. 1998). For example, molinate has been measured in the MIA at 42 µg/L (Julli and Krassoi 1995). More

than 100,000 kg is applied in each season in the Murrumbidgee Irrigation Area which exceeds any other herbicides used in the area (Bowmer et al. 1998). The bioavailability and toxicity of molinate to freshwater fish, shrimps, cladoceran and midges were studied by Phyu et al. (2004, 2005, 2006). The toxicities of molinate were compiled on non-targeted invertebrates (Burdett et al. 2001), cladocerans (Foster et al. 1998; Julli and Krassoi 1995) and phytoplankton (Sabater and Carrasco 1998), Development of resistance to molinate was also studied on cladocerans (Sanchez et al. 2004) and native Australian fish (Harford et al. 2005),

Chlorpyrifos, is the second most heavily applied pesticide in Australia (Radcliffe 2002). It is an organophosphorus insecticide, is highly toxic to animals and commonly used for control of pests in rice, and is aerially sprayed. Chlorpyrifos concentrations in Australian waterways are generally below 1 µg/L. However there are a few occasions where the concentrations were 26 µg/L in northern rivers and 25 µg/L in irrigation drainage close to rice fields in southern NSW (NRA 2000). The half-life of chlorpyrifos in natural waters is short and estimated at 16 to 33 hours (Marshall and Roberts 1978). Chlorpyrifos inhibits the enzyme acetylcholine esterase in animals (Straus and Chambers 1995). The acute toxicity of chlorpyrifos has been fairly well studied and data are available on cladocerans (Cáceres et al. 2007), chronic toxicity on *D. carinata* (Zalizniak and Nugegoda 2006), fish (Patra et al. 2007), and algal species (Bengtson et al. 2005; Van Donk et al. 1992).

An additional factor for their selection was that all three chemicals i.e atrazine, molinate and chlorpyrifos are widely being used in the same regions to control agicultural pests and therefore are likely to occur as mixtures. The three chemicals have in fact been recorded in the water ways of Australian agricultural areas (Thomas et al. 1998).

In terms of the toxicity of mixtures, chemicals with the same mechanism of action invariably conform to additivity while chemicals with different mechanisms of action can conform to additivity, synergism or antagonism. All three of the test chemicals have different mechanisms of action. Thus, the selected test compounds would permit

the investigation of the effect that chemicals with different modes of action will have when combined with elevated salinity and chemicals with other modes of action.

Information on the toxicity of mixtures of pesticides is well documented (Faust et al. 1994; Faust et al. 2001; George and Liber 2007; Junghans et al. 2006; Scholz et al. 2006; Wendt-Rasch et al. 2004). However, mixture toxicity studies among the selected three pesticides are limited to certain aspects, i.e., atrazine and chlorpyrifos on invertebrates (Belden and Lydy 2000; Pape-Lindstrom and Lydy 1997; Wacksman et al. 2005).

1.4. Interactions of salinity and pesticide toxicities

Pesticide pollution and elevated salinities occur together in many situations, i.e., waterways located adjacent to agricultural/irrigated lands. Thus, studies on the combined effects of pesticides and salinity provide an ecologically more realistic picture of impacts on the aquatic biota.

Except for a few studies, the combined effects of salinity and pesticides on aquatic organisms have not been adequately studied. Hall and Anderson (1995) compiled the available information on the influence of salinity on the toxicity of different chemicals, which included pesticides on freshwater, estuarine and marine species. They reviewed a range of test organisms including cladoceran and algal species. The review was done on the basis of salinity ranges on different chemical classes. However, the species studied had one or more life stages in Chesapeake Bay. Herbicides (including atrazine) and organophosphorus insecticides were tested against estuarine organisms. Herbicide toxicities were least in mid range salinities i.e., 15 ppt and organophosphorus insecticide toxicities increased at higher salinities above 20 ppt. Most of the studies focused almost entirely on estuarine animals, which are adapted to variations in salinity and thus not relevant to freshwater organisms (Hall et al. 1997; Hall and Anderson 1995; Hall et al. 1995). An information gap thus exists on the toxicity of pesticide and salinity mixtures to freshwater organisms.

The physiological effects of mixtures of pesticides and salinity may vary with the mode of action of pesticides and the organism of concern. The mixture of atrazine and chlorpyrifos to invertebrates is synergistic and the reason is that atrazine induces the formation of more toxic metabolites of chlorpyrifos (Pape-Lindstrom and Lydy 1997). The combined effect of molinate and atrazine could be more that the effects of individual chemicals. This could be due to inhibition of photosynthesis by atrazine make cells more vulnerable or kill (Solomon et al. 1996) and then inhibition of cell division by molinate (Tomlin 1994) may further inhibit the algae.

The molinate consists of neurotoxic effects on the animals and this may cause enhanced effects in the presence of other chemicals i.e. chlorpyrifos and atrazine.

Studies have revealed that exposure to elevated salinity affects osmoregulation in algae as well as changing their membrane permeability (Reed 1984). As a result it affects physiological functions such as photosynthesis (Allakhverdiev et al. 2000). When the salinity and the pesticides occur as mixtures the physiological effects could be further elevated.

In the case of cladocerans external salinities higher than that of the internal body fluid and haemolymph can cause mortality. As physiological functions are disrupted at higher salinity the combined effects of pesticides and salinity could be much more severe.

It is important to study the joint activities of highly toxic substance like pesticides and physiological stressors like salinity as salinity acts as a physiological stressor at low concentrations and as a toxicant at higher concentrations (Kefford et al. 2002). The outcome of such studies would assist in protecting aquatic organisms from the combined toxic effects of salinity and pesticides.

1.5. Toxicity of mixtures

1.5.1. Significance of pesticide mixtures

In aquatic environments, compounds do not exist in isolation but rather are generally found as mixtures. The toxicity of mixtures of pesticides has attracted growing concern among researchers, since the combined effects of such mixtures are not fully known. The interactive toxicity of chemical mixtures is very important in ecotoxicological studies (Belden and Lydy 2000; Broderius and Kahll 1995; Faust et al. 2000; Hall and Anderson 1995; Marking and Dawson 1975; Van den Brink et al. 1995) However, studies on mixture toxicity are relatively limited. One reason for this could be "the complexity of experimental design and the large number of tests required for exhaustive examination of even fairly simple mixtures" (ECETOC 2001).

1.5.2. Evaluation of pesticide mixtures

A number of schemes and models has been developed for the evaluation and prediction of toxic relationships of mixtures. Bliss (1939) proposed that chemical constituents of a mixture can elicit similar action, dissimilar action, or interaction. Plakett and Hewlett (1952) refined this view and further categorised possible joint actions (Table 1.6).

Table 1.6: The four types of joint actions for mixtures developed by Plackett and Hewlett (1952).

	Similar Joint Action	Dissimilar Joint Action
Non-interactive	Simple similar (concentration addition, CA)	Independent (response addition, RA)
Interactive	Complex similar	Dependent

The toxic unit (TU) approach proposed (Brown 1968; Sprague 1968) dicribes that the concentration of the toxicant is expressed as a proportion of its 50% effect concentration. So the toxic unit is derived by dividing the concentration by the EC50 value using the equation,

$$TU_i = \frac{C_i}{EC_{p_i}} \qquad (1)$$

where the subscript denotes the component 'i' of a mixture, while C_i is the aqueous concentration of component 'i' in a mixture and ECp_i is the aqueous concentration of the component acting individually, which will cause a given toxic effect (e.g., EC/IC50).

Marking (1977) proposed an additivity index for mixture toxicity evaluation. The toxic units of the components in the mixtures are added to express the total toxicity of the mixture. The treatments for the mixture toxicity experiments can then be expressed in TUs and the 50% effect can be derived using an appropriate method i.e., the US EPA Linear Interpolation method (Norberg-King 1998) or the Trimmed Spearman-Karber method (Hamilton et al. 1977). If the sum of the TU values for components of a mixture is 1 then the toxicity relationship is considered to conform to additivity. A deviation of less than 30% from additivity is considered as conforming to additivity while a deviation of greater than 30% is considered to conform to either antagonism or synergism (ECETOC 2001). This is graphically illustrated in Figure 1.2.

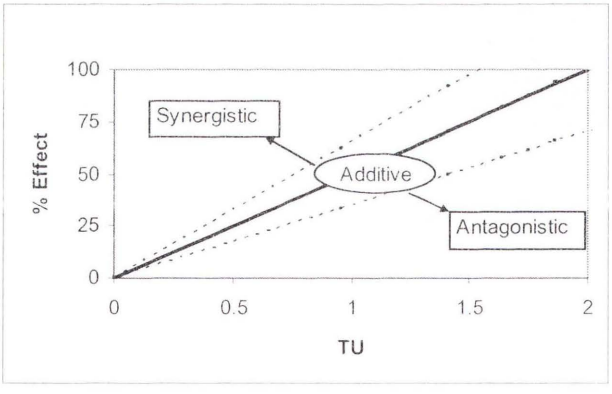

Figure 1.2: An example of the plots used to indicate the type of toxicity interaction that occurs within the mixture. The concentrations of the mixture (expressed as toxic units, TUs) that caused a certain % effect is plotted. The solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

There are two main inherent features of the concentration addition model (CA). First, approximately 10 – 30% of mixtures (irrespective of the type of chemical) are antagonistic or synergistic, with each type of joint action being equally frequent, and about 70 - 90% are additive (Deneer 2000; Faust et al. 1994; Ross and Warne 1997; Warne and Hawker 1995). Second, the CA model overestimates the effects and produces slightly higher estimates of the toxicity of mixtures than independent action (IA) where the chemicals have different mechanism of actions MeOAs (Backhaus et al. 2000a; Backhaus et al. 2000b; Chevre et al. 2006; Dyer et al. 2000; Faust et al. 1994; Junghans et al. 2006).

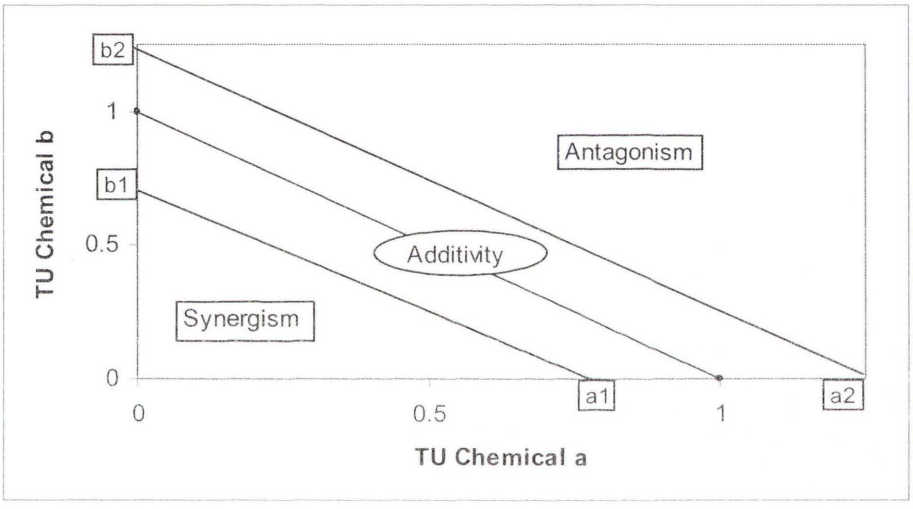

Figure 1.3: An example of the isobolographic plot used to indicate toxicity relationships i.e., additivity, antagonism and synergism. (a1 and a2 are the lower and upper confidence limits of EC/IC50 of chemical a, b1 and b2 are the lower and upper confidence limits of EC/IC50 of chemical b. The band demarcated by a1, b1, b2 and a2 is termed the additivity band. Mixture toxicity EC50/IC50 values that lie outside are statistically considered as being either antagonistic or synergistic.

The most common way of presenting mixture toxicity data graphically is using an isobologram. This is a two dimensional graph with the concentrations (expressed in terms of TU) of chemical 'a' and 'b' as the axes. The isobole linking the values on the y and x axes with values of 1 TU is the line of concentration addition (Figure 1.3). The EC/IC50 of single chemicals is also plotted on respective axis with their confidence limits. The upper confidence limit of a and b and the lower confidence limit of a and b are linked. The TU values of non-equitoxic mixtures that cause the

selected toxic effect (e.g., EC/IC50) are plotted on the isobologram (Warne 2003). If the values that cause the selected toxic effect lie above and to the right of the additivity line outside the confidence interval line then the mixture is antagonistic. If the values that cause the selected toxic effect lie below and to the left of the additivity line outside the confidence interval line then the mixture is synergistic. Values that are within the two confidence interval lines are additive (Altenburger et al. 1990; Warne 2003).

The limitations of this method are that the isobolographic method is only suitable for binary or tertiary mixtures because of the complexity of the experimental design, and the number of tests required to evaluate just one mixture. Therefore this approach is really only practical for rapid toxicity test methods such as the Microtox® bioassay. Another key limitation is the method used to determine if mixtures conform to antagonism, additivity or synergism. The reason for this is that the poorer the quality of the toxicity value (i.e., the larger the confidence interval) the more likely it will be considered to be additive. Therefore, a different method recommended by ECETOC (2001) was used to determine the toxicity relationship.

Simple mixtures (<5 compounds) show highly variable toxicities (anything from highly antagonistic to highly synergistic); however, complex mixtures (> 5 compounds) generally showed additive toxicity (Warne 1991). Based on this concept, Warne and Hawker (1995) put forward the Funnel hypothesis, which states that as the number of components in a mixture increases there is an increased tendency for toxicity to be additive. Ross and Warne (1997) working on mixtures of chemicals with different MeOAs (973 mixtures) found that the mixtures concurred with this hypothesis.

1.5.3. Water quality guidelines and pesticide mixtures

The general lack of mixture toxicity data and the fact that the toxicity of a mixture can vary with the relative concentrations of its components has meant that in the past environmental water quality guidelines in most countries have not incorporated mixture toxicity data. The Australian and New Zealand Water Quality Guidelines

(WQGs) (ANZECC and ARMCANZ 2000) did not incorporate the toxicity of mixtures in the derivation of trigger values but it was incorporated into the process of implementing the guidelines. Recently, the toxicity of herbicide mixtures has been incorporated into the WQGs of Quebec, Canada (Ministère de l'environment du Québec 2001) and a mechanism to include herbicide mixture toxicity into the Swiss water-quality guidelines was proposed by Chevere (2006).

Therefore, the establishment of baseline information on the combined toxic effects of salinity and mixtures of pesticide would serve an important purpose in further refining WQGs.

1.6. Exposure duration for different toxicants

The duration of exposure by organisms to toxicants plays an important role in modifying toxicity. Generally, as the duration increases the concentration at which toxic effects first commence decreases (Rand 1995). In the case of toxicants like salinity, exposure is very long-term, in the order of generations. With such long exposures there is always the chance that the exposed organisms will develop either genetically or physiologically based tolerance to salinity. There are examples of development of tolerance as a result of long-term exposure i.e., to metals (Gale et al. 2003; McLaughlin and Smolders 2001; Smolders et al. 2004; Tsui and Wang 2007). Therefore, short-term acute tests, which measure effects over a small portion of one generation provide little relevant information. Chronic tests are better but provide information on exposure for about a third of the lifespan of cladocerans. Life history tests provide information based on the exposure of test organisms over their entire lifespan. Multigenerational experiments provide a complete picture of the effects of toxicants on the individual and at the population-level life-history traits over exposure periods relevant to salinity. Therefore, it is important that multigenerational toxicity tests are conducted in order to understand the effects of salinity on aquatic organisms. However, time constraints and the enormous amount of resources required often limit such multigeneration toxicity experiments.

Exposure of aquatic organisms to toxicants such as pesticides is different from that of salinity exposure. The concentrations of pesticides in the environment depend on the spray pattern, their persistence and bioavailability amongst other factors; they are therefore not static over long periods. In most cases pesticides occur in the environment as pulses in relation to spray events over the growing season of a particular crop. So the bioavailable fraction of the chemical may be elevated for a short period and acute or chronic toxicity tests probably provide a reasonable estimate of likely toxic effects under such exposure scenarios.

1.7. Effects on community-level exposure

Single-species toxicity tests provide information only for the species in question and cannot be extrapolated to the community-level. If there is more than one species representing different trophic levels covering primary producers, primary consumers, etc., community-level information would be much more useful in evaluating community-level effects. One difficulty relating to these tests is that species representing different trophic levels cannot be tested in the same test container as predators will eat the prey. This is why mesocosms invariably do not contain fish unless they are physically separated by barriers (Cardinale and Palmer 2002). Further, the cost and practical difficulties associated with mesocosm systems limit the ability of researchers to conduct such studies frequently. For these reasons the OECD (1992) provides guidance on when such tests should be conducted and the USEPA has withdrawn funding for assessing pesticide impacts in mesocosms (Bradbury *pers comm*). Therefore, the majority of toxicity data will, in the near future, continue to be generated from single species tests and it is therefore vital that any potential interactive effects are considered when interpreting or using single species toxicity data. This should be possible if single species toxicity tests are conducted using representatives of different trophic levels (e.g., alga representing primary producers and cladocerans representing primary consumers). The two test species *D. carinata* and *P. subcapitata* are commonly found in NSW, Australia (Benzie 1986; Kobayashi et al. 2005) and are relevant for the study since they should co-exist in the same environment.

1.8. Aim and objectives of the study

The aim of the present study was to determine the toxic effects caused by salinity and pesticides individually and their mixtures on a representative freshwater primary producer - the alga *Pseudokirchneriella subcapitata* and a primary consumer - the cladoceran *Daphnia carinata*.

The specific objectives of the study were:

- To assess the population level effects and life-history traits of long-term exposure to elevated salinity on *P. subcapitata* and *D. carinata*;

- To determine the toxicity of elevated salinity and three selected pesticides, atrazine, molinate and chlorpyrifos, individually and in mixtures to *P. subcapitata* and *D. carinata*;

- To determine the toxicity of elevated salinity and three selected pesticides, atrazine, molinate and chlorpyrifos, individually and in mixtures on *P. subcapitata* and *D. carinata* that had been acclimatised to elevated salinity for several generations; and to determine if the multigeneration acclimatisation changed the organisms' sensitivity to salinity and pesticides acting individually and in mixtures; and

- Through the above studies to develop an understanding on potential effects of increased salinity by itself and in combination with pesticides on Australian freshwater organisms and to provide baseline information for refining water quality guidelines.

Chapter 2

Effects of multigenerational exposure to salinity on life history traits of the freshwater alga *Pseudokirchneriella subcapitata*

2.1. Abstract

Salinisation has been identified as a major threat to freshwater ecosystems. While many studies have focused on the effects of salinity on freshwater biota, none have examined the effects of exposing primary producers to elevated salinity for multiple generations. In the present study, the growth rate, number of cell divisions per day, generation time and cell yield of the unicellular green alga *Pseudokirchneriella subcapitata* exposed to elevated salinities of 3000 and 6000 µS/cm over five successive 72 hour long (C0, C1, C2, C3, C4) cultures (or approximately twenty cell doublings) were studied. Except for the second culture (C1), both elevated salinities had significant ($p \leq 0.05$) adverse effects on the growth rate, number of cell divisions per day and generation time of *P. subcapitata*.

Significant differences ($p \leq 0.05$) between cultures were observed (compared with the C0 culture) in terms of the growth rate, cell divisions per day and generation time indicating that stress induced by the elevated salinity persisted over the five cultures. There was no significant difference ($p > 0.05$) in cell yield between the three treatments in the first culture but in the second to fifth cultures both the elevated salinity treatments had cell yields significantly lower ($p \leq 0.05$) than that of the controls.

The observed effects on the life history parameters are probably due to salt induced effects on physiological functions such as osmoregulation, photosynthesis and metabolism of the alga. There could be direct toxic effects by individual ions to a lesser extent, since monovalant ions are dominant in sea water. The results show that the exposure of *P. subcapitata* to elevated salinity for a period sufficient to permit

approximately twenty cell doublings could adversely affect algal communities which in turn could affect the viability of aquatic food chains.

2.2. Introduction

The salinisation of inland aquatic systems has become a major environmental problem in some parts of the world and the situation is significant in Australia (Jolly et al. 2001; Williams 1987). Although natural salinisation of landscapes does occur in certain localities this has been accelerated through human activities (Jolly et al. 2001). In Australia the accelerated rise in salinity in inland rivers and wetlands has occurred over 200 years since European settlement due to land clearing and agricultural practices. Pinder et al. (2005) reported salinities in inland water bodies in Western Australian wheat belt to range from <4300 to 140,000 μS/cm, while Jolly et al. (2001) reported a mean increase of 4.37 μS/cm/year with a minimum of -6.9 to a maximum of 139.5 μS/cm/year in streams in the Murrumbidgee Irrigation Area.

The effects of salinity on Australian freshwater ecosystems are fairly well documented (Halse et al. 2003; Halse et al. 1998; Hart et al. 1991; James et al. 2003; Kefford 1998; Kefford et al. 2006; Kefford et al. 2005; Kefford et al. 2004; Kefford et al. 2003). Most of these studies have focused on freshwater invertebrates, but the aquatic plants, especially algae, have not been adequately studied. Green algae play an important role as primary producers in inland Australian aquatic systems to support food webs and maintain ecosystem functioning (Douglas et al. 2005). Therefore, it is necessary to understand the impacts of rising salinity on such species.

In agricultural areas organisms living in such saline systems would also have been exposed to the gradual increase in salinity over this relatively long period. Such long-term exposures should permit organisms to adapt to the rising salinity. Thus, acute and chronic toxicity experiments cannot provide a realistic picture on the life history traits of these organisms. Multigeneration studies provide more relevant and useful population level information.

There is no published information on the effects of multigenerational exposure of freshwater organisms to salinity. Therefore, it would be pertinent to determine such effects which are essential for conservation measures of salt affected environments. The objectives of the present study were:

- To determine multigenerational effects of salinity on the life history traits of the green alga *P. subcapitata* exposed to elevated salinity over five successive cultures (i.e., approximately 20 generations).

- To determine whether there are changes in sensitivity of *P. subcapitata* to salinity as a result of exposure to elevated salinity over five successive cultures (i.e., approximately 20 generations).

2.3. Materials and Methods

2.3.1 Test species

The green alga *Pseudokirchneriella subcapitata* Hindak, formerly known as *Selenastrum capricornutum*, is a unicellular non-motile crescent-shaped (40 – 60 μm^3) alga that belongs to the family Chlorophyceae. The algal culture (American Type Culture Collection [ATCC No. 22662]) used in the present study was originally obtained from the Centre for Advanced Analytical Chemistry, CSIRO. This species was selected as it: represents primary producers and as such acts as an important food source for herbivorous consumers such as cladocerans; it is easy to culture in the laboratory; it is extensively used in ecotoxicology; clumping of cells or formation of chains rarely occurs thus enabling accurate enumeration using an electronic particle counter; it grows sufficiently rapidly to permit an accurate measurement of cell yield after 72 hours; and it is moderately sensitive to a wide variety of toxic substances (Environment Canada 1997).

2.3.2. The toxicant

Sea water collected from Cronulla, NSW, was used to adjust the salinity of the laboratory water. Sea water was used as the ionic composition of Australian inland salt is similar to that of sea water (Bayly and Williams 1972).

2.3.3. Algal cultures

Stock cultures (plate cultures) of *P. subcapitata* were used to commence liquid algal cultures by aseptically transferring a loopful of algal culture to 100 mL of liquid culture medium (Table 2.1) in 250 mL sterile flasks. The liquid stock culture was grown on a shaker table in an algal culture room under continuous 'cool-white' fluorescent lighting with an intensity of 4000 lux or 65 ± 5 $\mu E\ s^{-1}m^{-1}$ at the surface of the cultures and at 24 ± 1°C.

The stock culture was renewed weekly by aseptically transferring 1 to 2 mL of the previous week's culture into a 250 mL sterile flask containing 100 mL of algal culture medium. The new stock cultures were incubated as described above.

In order to prepare the liquid culture medium five stock nutrient solutions listed in Table 2.1 were prepared using reagent grade chemicals and polished deionised water. Then 1 mL of each stock nutrient solution was added to 900 mL of Milli-Q water in a volumetric flask. The volume was made up to 1 L with Milli-Q water, mixed well, and the pH adjusted to 7.5 ± 0.1 using 0.1 M NaOH or HCl as appropriate. The final concentrations of the nutrients followed those of the US EPA (1994b) and Environment Canada (1997) culture media for *P. subcapitata*. The salinity of algal culture media is 100 µS/cm and this salinity is a result of the addition of all the nutrients which provide optimum conditions for the algal growth. Therefore the salinity of the algal control cultures was 100 µS/cm and this was referred to as the 'normal salinity' for *P. subcapitata*.

The new culture medium was immediately filter-sterilised through a 0.22 μm membrane filter into a pre-autoclaved 1 L Schott bottle. The filter sterilization process was undertaken in a laminar flow cabinet using a sterile vacuum and pre-sterilised disposable 0.22 μm filters. This sterile medium was kept in the fridge at 4°C when not in use to avoid contamination (Environment Canada 1997).

A monthly check on the quality of *P. subcapitata* in the cultures was performed by microscopic examination. The cultures were examined in terms of cell morphology, colour, clumping of cells, absence of bacteria or yeast, and measuring their relative sensitivities to a reference toxicant (sea water).

Table 2.1: Nutrient stock solutions used for the algal culture medium and for the dilution water used in toxicity tests (Environment Canada 1997).

Stock solution	Chemical compound
1	$MgCl_2 \bullet 6H_2O$
	$CaCl_2 \bullet 2H_2O$
	H_3BO_3
	$MnCl_2 \bullet 4H_2O$
	$ZnCl_2$
	$FeCl_3 \bullet 6H_2O$
	$CoCl_2 \bullet 6H_2O$
	$Na_2MoO_4 \bullet 2H_2O$
	$CuCl_2 \bullet 2H_2O$
	$Na_2EDTA \bullet 2H_2O$
2	$NaNO_3$
3	$MgSO_4 \bullet 7H_2O$
4	K_2HPO_4
5	$NaHCO_3$

2.3.4. Experimental design

The procedure used for the multigeneration experiment was similar to the 72 hour growth inhibition test used by the USEPA (1993) and Environment Canada (1992). Modifications were that 6 mL vials were replaced by 200 mL conical flasks and rather than discarding the cultures after 72 hours they were used to start the next culture at its corresponding salinity.

The multigenerational tests consisted of two salinity treatments (3000 and 6000 µS/cm) and a control (with a salinity of 100 µS/cm - the salinity of the algal medium). The salinity of the treatments was selected based on their relevance to field salinity conditions in Australian inland waters (Kefford et al. 2004, Pinder et al. 2005) and based on the results of chronic salinity toxicity tests. Since the endpoint should be based on the inhibition concentration it was decided not to exceed 60% inhibition of algal growth in order to exert sufficient toxic effect and to have sufficient viable cells to complete the multigeneration tests. At the salinity level of 6000 µS/cm there were about 55% inhibition of growth on average (based on the chronic test results conducted for salinity reference toxicity tests, refer Chapter 3).

There were five replicates for each treatment. These test media were prepared by mixing appropriate amounts of filtered (by 0.22 µm filter), UV-sterilised and aerated seawater with the Milli-Q water prior to the addition of the nutrient solutions described in Table 2.1. The test volumes were 100 mL contained in 200 mL conical flasks. In addition, two replicates each of a control blank and sample blank which consisted of diluent water and test solution without algae were prepared. Of the five replicates of each salinity treatment, four were used for determining cell yield at the end of the test, while the fifth replicate was used for measuring physico-chemical parameters (temperature, pH, salinity and dissolved oxygen) of the test solutions at the beginning and end of the test.

To initiate the multigeneration tests, a four- to seven-day old culture of exponentially growing algae was washed to remove the old culture medium. This was done by centrifuging, decanting and resuspending the algal cells in new algal medium four

times, the last resuspension being in approximately 20 mL of new algal medium. The density of the algal suspension (the inoculum) was determined and the volume required for 1 x 10^4 cells/mL to be inoculated into the 100 mL test solutions was calculated. The calculated volume of inoculum was added to each of the test solutions but not to the blank solutions. The inocula were thoroughly mixed and cell densities in each flask were measured and recorded. Then all solutions were incubated at 24 ± 1°C for 72 hours on a shaker table under cool white light with a light intensity of 4000 lux at the surface of the test solution.

After 72 hours, the cell densities in all the test flasks were counted using an electronic particle counter (LiQuilaz-E20 with a liquid sampler LS200 and a software package APSS-View21 CFR11). At this time each existing culture was terminated and new cultures were started using the corresponding previous cultures prior to being terminated and cell densities of the new cultures were recorded. *P. subcapitata* were exposed to the above salinity treatments in five successive cultures starting with culture one (C0) and four subsequent cultures (i.e., C1, C2, C3 and C4). The growth rates, doublings per day and generation time were calculated for each culture using the recorded cell densities at the beginning and end of each culture.

The growth rate (K'), number of cell divisions per day and the generation time (T) were calculated using following equations (CSIRO 2005).

$$K' = Ln (N2 / N1) / (t2 - t1) \quad (1)$$

where N1 and N2 = biomass at time 1 (t1) and time 2 (t2) respectively.

$$\text{cell divisions per day} = K' / Ln2 \quad (2)$$

$$T = 1 / \text{no. divisions per day} \quad (3)$$

2.3.5. Statistical analysis

The data were tested for homogeneity and normality using Bartlett's test and Chi squared tests. If the data did not meet these assumptions they were appropriately transformed before further analysis. The data were analysed using ANOVA and subjected to pair-wise analysis using Dunnett's test to compare variations between treatments in each culture (Meyer et al. 1987). Based on this, the concentrations statistically different to that of the controls of each culture were determined. Differences between cultures were analysed using the standard error of the difference test (Sprague and Fogels 1977).

2.4. Results

The ranges of each physicochemical property measured in all treatments of all the toxicity tests were within the acceptable limits. The variations in the growth rates, divisions per day and generation time are graphically illustrated for each culture in Figures 2.1 – 2.5.

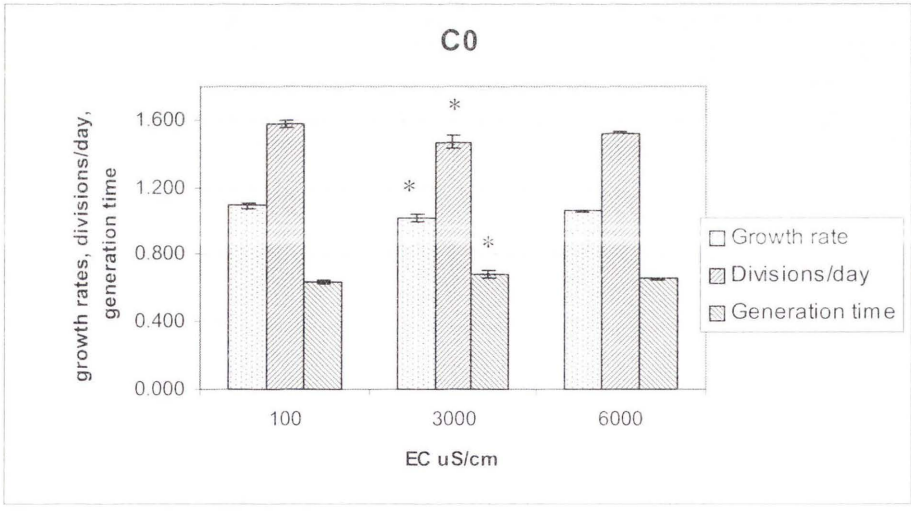

Figure 2.1: Variations in mean growth rate, cell divisions per day and generation time in the C0 culture of *Pseudokirchneriella subcapitata*. [Error bars: standard error of the means, * indicates endpoint significantly different ($p \leq 0.05$) to that of the control (100 µS/cm)].

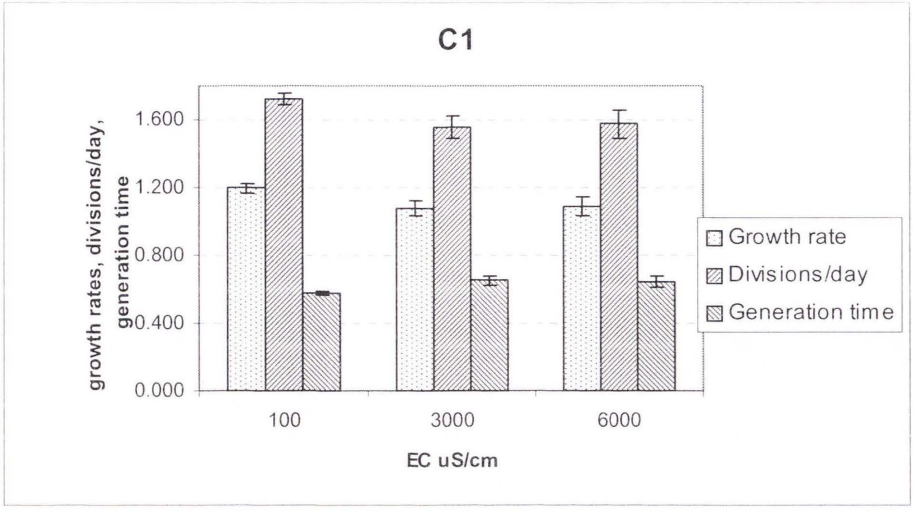

Figure 2.2: Variations in mean growth rate, cell divisions/day and generation time in the C1 culture of *Pseudokirchneriella subcapitata* [Error bars: standard error of the means].

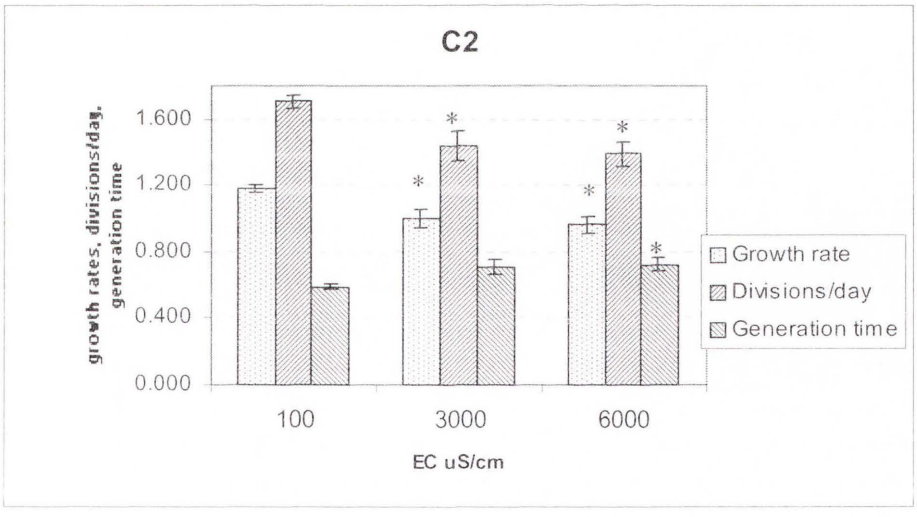

Figure 2.3: Variations in mean growth rate, cell divisions/day and generation time in the C2 culture of *Pseudokirchneriella subcapitata* [Error bars: standard error of the means, * indicates endpoint significantly different ($p \leq 0.05$) with that of the control (100 µS/cm)].

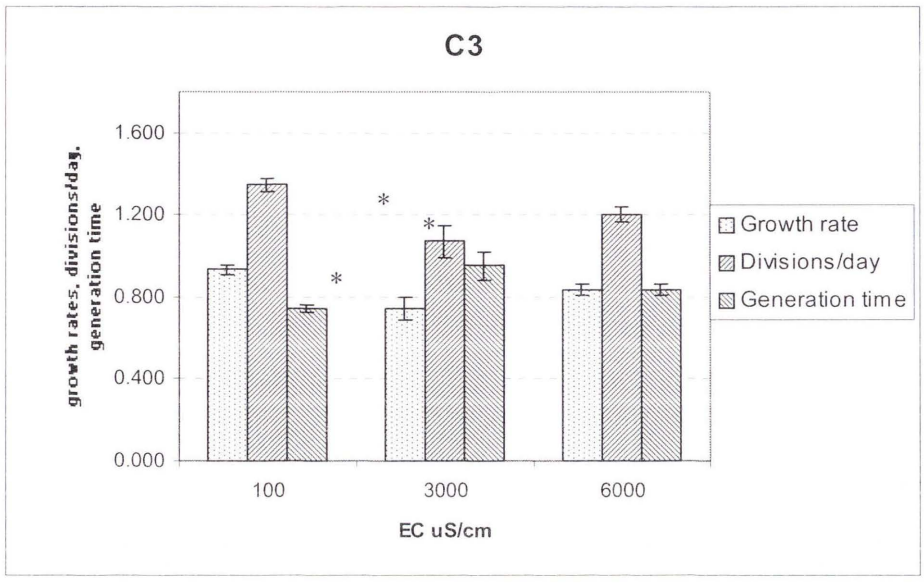

Figure 2.4: Variations in mean growth rate, cell divisions/day and generation time in the C3 culture of *Pseudokirchneriella subcapitata* [Error bars: standard error of the means, * indicates endpoint significantly different ($p \leq 0.05$) with that of the control (100 µS/cm)].

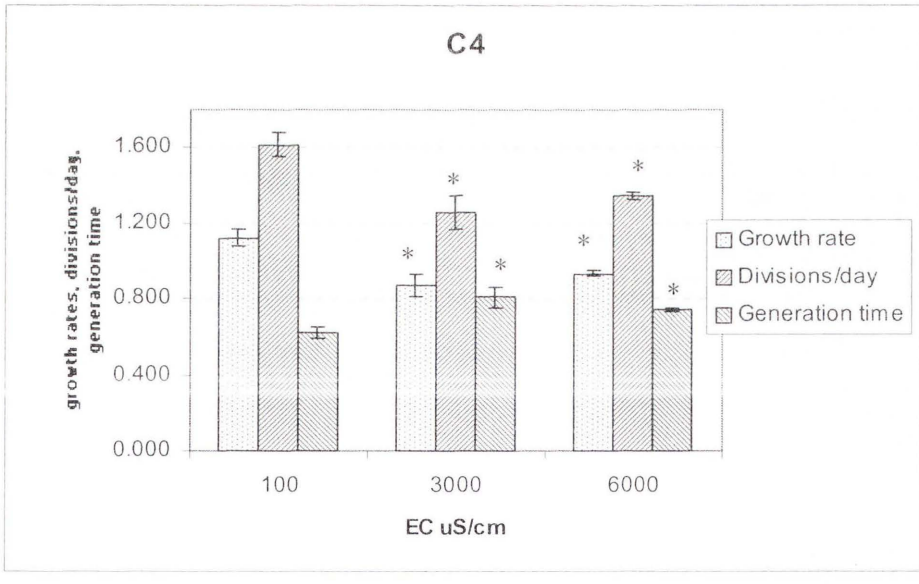

Figure 2.5: Variations in mean growth rate, cell divisions/day and generation time in the C4 culture of *Pseudokirchneriella subcapitata* [Error bars: standard error of the means, * indicates endpoint significantly different ($p \leq 0.05$) with that of the control (100 µS/cm)].

In the C0 culture the 3000 µS/cm treatments growth rate and cell divisions per day were significantly lower ($p \leq 0.05$) and generation time was significantly higher ($p \leq 0.05$) than that of the control. None of the parameters in the 6000 µS/cm treatment were significantly different ($p > 0.05$) from those of the control (Figure 2.1). None of the parameters were significantly different ($p > 0.05$) to those of the control in the C1 culture (Figure 2.2).

In the C2 culture, growth rate and cell divisions/ day at 3000 µS/cm and all three parameters at 6000 µS/cm were significantly different ($p \leq 0.05$) to those of the control (Figure 2.3). The three parameters at 3000 µS/cm were significantly different ($p \leq 0.05$) in the C3 culture but no parameters were significantly different ($p > 0.05$) to those of the control at 6000 µS/cm (Figure 2.4) while all three parameters in the C4 culture at both 3000 and 6000 µS/cm were significantly ($p \leq 0.05$) different to those of the control (Figure 2.5).

There were no significant ($p > 0.05$) differences in cell yield in the C0 culture (Table 2.2). However, in all subsequent cultures (i.e., C1 to C4) cell yields were significantly lower ($p \leq 0.05$) for both elevated salinities (i.e. 3000 and 6000 µS/cm) compared to that of the controls (Table 2.2).

Table 2.2: Statistical comparisons of the *Pseudokirchneriella subcapitata* cell yield (cells/100 mL) ± SE in the 3000 and 6000 µS/cm salinity treatments with that of the controls (i.e., 100 µS/cm) for each culture.

Cultures	Control 100 µS/cm	3000 µS/cm	6000 µS/cm
C0	174.9 ± 9.3	143.9 ± 17.4	157.8 ± 4.0
C1	174.6 ± 15.0	108.5* ± 22.7	98.8* ± 16.3
C2	151.5 ± 29.8	56.6* ± 12.8	53.9* ± 7.5
C3	171.2 ± 13.3	51.2* ± 10.4	52.1* ± 5.2
C4	143.4 ± 17.4	41.8* ± 9.2	49.4* ± 5.8

*= significantly different ($p \leq 0.05$) to the cell yield of the control.

Table 2.3 summarises the values for growth rate, divisions per day, generation time and cell yield for each culture and treatment and statistical comparisons of the parameter values for each culture (i.e., C1 to C4) with those of the C0 culture.

In the control treatment (100 μS/cm), except for the C4 culture, other cultures (C1, C2 and C3) were significantly different ($p \leq 0.05$) to the C0 culture in terms of the growth rate, cell divisions per day and generation time. The C4 culture was significantly ($p \leq 0.05$) lower than that of C0 culture for cell yield, but the C1 – C3 cultures were not significantly different ($p > 0.05$) from the C0 culture

The growth rate at 3000 μS/cm was significantly greater ($p \leq 0.05$) in the C1 culture, and significantly smaller ($p \leq 0.05$) in the C3 and C4 cultures and in the 6000 μS/cm treatment the C2, C3 and C4 cultures were all significantly smaller ($p \leq 0.05$) than that of the C0 culture.

Significant increases ($p \leq 0.05$) in cell divisions per day were observed in the C1 culture, but in the C3 and C4 cultures significant ($p \leq 0.05$) decreases occurred at 3000 μS/cm compared with that in the C0 culture. For the 6000 μS/cm treatment cell divisions per day in the C2, C3 and C4 cultures were all significantly smaller ($p<0.05$) than that in the C0 culture. Generation time significantly increased ($p \leq 0.05$) in the C2, C3 and C4 cultures at both the 3000 and 6000 μS/cm treatments compared with that in the C0 culture.

Table 2.3: The growth rate, cell divisions per day and generation time of *Pseudokirchneriella subcapitata* in the three treatments for each culture.

Parameter	Salinity treatment (µS/cm)	C0	C1	C2	C3	C4
Growth rate (Cells per day)	100	1.09 (1.07 - 1.11)	1.20* (1.17 - 1.29)	1.18* (1.16 - 1.21)	0.93* (0.91 - 0.96)	1.12 (1.07 – 1.17)
	3000	1.02 (0.99 - 1.05)	1.08* (1.03 - 1.13)	1.00 (0.94 – 1.06)	0.74* (0.69 - 0.80)	0.87* (0.81 – 0.93)
	6000	1.06 (1.05 - 1.06)	1.09 (1.03 - 1.15)	0.96* (0.91 – 1.01)	0.84* (0.81 - 0.86)	0.93* (0.92 – 0.93)
Divisions (per day)	100	1.58 (1.55 - 1.60)	1.72* (1.69 - 1.76)	1.71* (1.67 – 1.74)	1.35* (1.32 - 1.38)	1.62 (1.55 – 1.68)
	3000	1.47 (1.43 - 1.51)	1.56* (1.49 - 1.62)	1.40 (1.36 – 1.53)	1.07* (0.95 - 1.00)	1.26* (1.17 – 1.35)
	6000	1.53 (1.52 - 1.53)	1.57 (1.49 - 1.66)	1.39* (1.32 – 1.46)	1.21* (1.17 - 1.24)	1.35* (1.33 – 1.36)
Generation time (days)	100	0.64 (0.63 - 0.65)	0.58* (0.57 - 0.59)	0.59* (0.58 – 0.60)	0.74* (0.73 - 0.76)	0.62 (0.60 – 0.65)
	3000	0.68 (0.67 - 0.70)	0.65 (0.62 - 0.68)	0.70* (0.66 – 0.75)	0.95* (0.89 - 1.02)	0.81* (0.75 – 0.86)
	6000	0.656 (0.65 - 0.66)	0.64 (0.61 - 0.68)	0.73* (0.69 - 0.76)	0.83* (0.81 - 0.86)	0.75* (0.74 – 0.76)
Cell Yield (Cells/100 Ml)	100	174.9 (165.6-184.2)	174.6 (159.6-189.7)	151.5 (121.8-181.3)	171.2 (158.0- 184.5)	143.4* (126.0-160.7)
	3000	143.9 (126.0-161.8)	108.5* (85.8 - 131.1)	56.6* (43.8 - 69.4)	51.2* (40.9 - 61.6)	41.8* (32.5 - 51.0)
	6000	157.8 (153.9-161.8)	98.8* (82.5 - 115.2)	53.9* (46.4 - 61.4)	52.1* (46.9 - 57.2)	49.4* (43.6 - 55.2)

* Significantly different ($p \leq 0.05$) from those of the C0 culture

Significantly smaller ($p \leq 0.05$) cell yields were observed in the C1 – C4 cultures in both salinity treatments (3000 and 6000 µS/cm) compared with that of the C0 culture.

2.5. Discussion

In terms of the growth rates, cell division rates and generation times the 3000 µS/cm treatment in the C0 and C3 cultures and both the 3000 and 6000 µS/cm treatments in the C2 and C4 cultures were significantly ($p \leq 0.05$) different from their respective controls. This indicates that elevated salinity adversely affected life history traits of

the alga *P. subcapitata* over the generations. Both salinity treatments in C1 and the 6000 µS/cm treatment in C0 and C3 were not significantly ($p > 0.05$) different from that of the controls. There are some possible explanations for these observations. Exposure to the toxicant could sometimes induce stimulatory effects at low concentrations, which is termed hormesis (Calabrese 2005; Kefford et al. 2008). The maximum response from hormesis is typically modest (30-60% greater than controls) and often affects different endpoints at different concentrations (Calabrese 2005). This was observed for different endpoints for salinity for freshwater insects (Hassell et al. 2006; Kefford et al. 2008). Another reason could be that there was a shift in the threshold salinity caused negative effects in multigenerational exposure to salinity. Such effects are dependent on the concentration of the toxicant as well as the number of generations that are exposed to the toxicant (Rose et al. 2002a) and found similar types of variations in the concentrations of chemicals at which adverse effects were observed on life-history traits.

There were no significant variations ($p > 0.05$) in algal cell yield (Table 2.2) among the treatments in the C0 culture, which indicates that healthy alga used to commence the tests were able to cope with exposure to salinity of 3000 and 6000 µS/cm. In all subsequent cultures algal populations in both high salinity treatments were significantly ($p \leq 0.05$) different from their corresponding controls. The commencement of effects on cell yield was rapid (i.e., within 72 hours). Thus after the first culture, a salinity of at least 3000 µS/cm would detrimentally affect life history traits of *P. subcapitata*. The persistence of the effects of elevated salinity over subsequent cultures indicate that while the alga can survive under these conditions that they do not develop tolerance to salinity.

There were significant variations between the cultures in terms of growth rates, cell divisions per day, generation times and cell yield (Table 2.3). This indicates a reduction in salt tolerance of the alga over several generations of exposure.

Elevated salinity affects osmoregulation in algae as well as changing their membrane permeability (Reed 1984). The changes in osmotic pressure of the cellular fluid of algal cells as a result of the high salinity in the external medium can lead to disruption

of physiological functions such as photosynthesis (Allakhverdiev et al. 2000). Inhibition of photosynthesis in turn, can affect all cellular functions due to a reduction of available energy. Elevated salinity has also been reported to affect phosphorus uptake and metabolism in algae (Rai and Sharma 2006). Even though the experiments in the present study were conducted under optimum nutrient conditions, it is clear that algal populations were negatively affected by increasing salinity to 3000 µS/cm. In the environment where nutrient conditions are unlikely to be optimal, even stronger effects may be experienced by algal populations.

Apart from osmosis related effects, individual ions in salt can have toxic effects on the algae (Cleave et al. 1981). The extent of adverse effects of metal ions depends on the ratio of monovalent to divalent ions (Cleave et al. 1981). The composition of Australian inland water is similar to that of seawater. The order of dominance of concentrations of cation and anions in sea water and in inland water bodies in Queensland is Na>Mg>Ca>K and $Cl^- > SO_4^- > HCO_3$ (Bayly and Williams 1972). The sea water that was used in the present study is dominated by less toxic monovalant ions (e.g., Na^+ and Cl^-) and therefore the extent of their toxic effects would not be as high as with other sources of elevated salinity such as mine effluent in which the divalent and heavy metal concentrations are higher (Kennedy et al. 2003).

2.6. Conclusions

The fact that exposure of *P. subcapitata* to salinity over several generations suppressed growth rates, cell division rates, and increased generation times shows that negative impacts on the viability of the alga in salt affected environments can occur at a salinity of 3000 µS/cm. Such impacts on the algae which are primary producers in aquatic environments in turn may affect the existence of species in higher trophic levels of food chains in the environment.

Generally, the effect of elevated salinity on the life history traits was greatest in the second and third cultures and then levelled off or showed slight recovery in subsequent cultures. However, for all traits the deleterious impact persisted for the full five successive cultures.

Chapter 3

Chronic toxicity of salinity and the pesticides atrazine, molinate and chlorpyrifos individually and as mixtures to the freshwater alga, *Pseudokirchneriella subcapitata*

3.1. Abstract

Many inland waterways are experiencing increasing salinity in conjunction with continuing pollution by pesticides associated with agriculture. The effects of salinity and pesticides individually have been studied and are well documented. However, there is no published information on the effects of mixtures of pesticides and salinity on freshwater algae. The present study determined the chronic toxicities (72h inhibition of growth) of salinity and three pesticides, atrazine, molinate and chlorpyrifos individually, in mixtures of individual pesticides and salinity, in equitoxic mixtures of pesticides, and in mixtures of the three pesticides and salinity to the freshwater alga, *Pseudokirchneriella subcapitata.*

The individual toxicities (72-hour IC50 values) of salinity, atrazine, molinate and chlorpyrifos to *P. subcapitata* were approximately 5600 μS/cm, 48 μg/L, 300 μg/L, 800 μg/L, respectively. Atrazine had a very high chronic toxicity to *P. subcapitata* while molinate and chlorpyrifos were highly toxic. The toxicity of the mixtures of atrazine and salinity, and of molinate and salinity conformed to additivity. In contrast, the mixture of chlorpyrifos and salinity conformed to antagonism. The equitoxic mixtures of pesticides and the mixtures of multiple pesticides with salinity conformed to additivity and antagonism approximately 50% each, while synergism accounted for less than 2%. Thus, sufficient protection for the freshwater algal species from the atrazine, molinate and chlorpyrifos mixtures in combination with salinity can be achieved if additivity of the test toxicants is considered in the development of water quality guidelines.

3.2. Introduction

Salinisation in inland Australia is a serious problem to freshwater environments. Salinisation affects freshwater organisms inhabiting such environments through toxic effects and physiological stress. The consequences of increasing salinity are well documented (Halse et al. 1998; Hart et al. 1991; James et al. 2003; Kefford 1998; Kefford et al. 2006; Kefford et al. 2005; Kefford et al. 2002; Kefford et al. 2004; Kefford et al. 2003; Nielsen et al. 2003). In Australia salinity is especially common in irrigated agricultural areas (Hart et al. 1991; Pinder et al. 2005). In such areas, there is also additional pressure on aquatic environments arising from pesticides used for crop protection.

The toxicity of single toxicants and mixtures of toxicants can adversely affect individual species and communities in aquatic environments. Evaluation of the effects of toxicants on primary producers such as unicellular algae is extremely important in determining the viability of such communities. The freshwater alga *Pseudokirchneriella subcapitata* has been used extensively to determine the effects of toxicants and stressors in aquatic environments (Phyu 2004; Rojickova-Padrtova and Marsalek 1999; Sabater and Carrasco 1998; Warne et al. 1998). However, information on the effects of salinity on freshwater primary producers is extremely rare. One example is the study of Cleave et al. (1980) on the toxicity of salinity and different ions on *Selenastrum capricornutum* (now called *P. subcapitata*) and *Synedra delicatissma*, which found that *P. subcapitata* was relatively more sensitive compared with *S. delicatissma* to a variety of salts. In contrast, the effects of pesticides on non-target species including freshwater algae have been extensively studied (Ma et al. 2006; Sabater and Carrasco 2001; Sancho 2003; Van Donk et al. 1992).

Information on the toxicity of mixtures of metals (Wong and Beaver 1980), dissimilarly acting chemicals (Faust et al. 2003), pesticides (Fairchild et al. 1994; Faust et al. 2001; Kungolos et al. 1999; Van den Brink et al. 1995), solvents and pesticides (Bailey et al. 2000; Jay 1996), and whole effluent (Bailey et al. 2000) to freshwater algae is available. However, the combined effects of salinity and

pesticides, and salinity and multiple pesticide mixtures have not been studied in relation to freshwater algae.

The objectives of this study were to determine the chronic toxicity of:

- atrazine, chlorpyrifos, molinate, and salinity individually to *P. subcapitata.*
- mixtures of these pesticides individually and salinity to *P. subcapitata;*
- equitoxic mixtures of these pesticides to *P. subcapitata*; and
- these pesticides as multiple mixtures and salinity to *P. subcapitata.*

3.3. Materials and Methods

3.3.1. Test species

The green alga *Pseudokirchneriella subcapitata* Hindak, formerly known as *Selenastrum capricornutum*, is a unicellular non-motile crescent-shaped alga (40–60 μm^3) belonging to the Family Chlorophyceae. The algal culture used in the present study (i.e., American Type Culture Collection [ATCC No. 22662]) was maintained at the UTS/NSW DECC Centre for Ecotoxicology laboratory and was originally obtained from the CSIRO Centre for Advanced Analytical Chemistry, Lucas Heights, NSW. This species was selected as it: represents primary producers as an important food source for herbivorous consumers such as cladocerans; is easy to culture in the laboratory; is extensively used as a test species in ecotoxicology; rarely has clumping of cells or formation of chains, thus enabling accurate enumeration using a particle counter; grows sufficiently rapidly to permit accurate measurement of cell yield after 72 hours; and is moderately sensitive to a range of toxic substances (Environment Canada, 1997).

3.3.2. Toxicants

The pesticides used in the study were atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1, 3, 5-triazine-2, 4-diamine; CAS No-1912-24-9), chlorpyrifos (O,O-Diethyl-O-(3,5,6-

trichloro-2-pyridinyl) phosphorothioate, CAS No. 2921-88-2) and molinate (s-ethyl *N*, *N*-hexa methylene thiocarbamate; CAS No-2212-67-1). All were all reagent-grade technical chemicals (≥ 97% purity). These pesticides were selected because they are among the most commonly used in Australian agriculture (Bowmer et al. 1998, Thomas et al. 1998). Stock and working solutions of the pesticides were prepared in analytical-grade (99% purity) acetone as the carrier solvent. The stock solutions were stored in a freezer at –4 °C and all working solutions were prepared immediately prior to use.

Sea water collected from Cronulla, NSW, was used as the source of salinity for all toxicity tests that required elevated salinities as the ionic composition of Australian inland salinity is essentially similar to that of sea water (Bayly and Williams 1972).

3.3.3. Algal cultures

Culturing of the algae was conducted according to the methods described in the (USEPA 1994a) and (Environment Canada 1992) documents. Details of the culture methods are described in Chapter 2.

3.3.4. Experimental design

The toxicity experiments for *P. subcapitata* were conducted in four groups.

3.3.4.1. Group 1 experiments

Group 1 experiments consisted of chronic toxicity tests with individual toxicants, i.e., salinity, atrazine, chlorpyrifos and molinate. The results of individual toxicities were used to determine the concentrations of pesticides to be used in the mixture toxicity tests.

3.3.4.2. Group 2 experiments

Group 2 experiments consisted of toxicity tests for mixtures of salinity and individual pesticides (i.e., salinity/atrazine, salinity/chlorpyrifos, and salinity/molinate). The mixtures were made using a single concentration of the pesticides (i.e., the

concentration that causes 50% inhibition of algal growth - IC50 value) with variable concentrations of sea salt (Table 3.1). Ideally the total TUs for each treatment of each mixture should be identical but small variations do occur. The TU values were based on measured concentration values of each pesticide in the mixtures (Table 3.1).

Table 3.1: The measured composition of each treatment of the individual pesticide and salinity mixtures expressed in terms of toxic units (TU) of the salinity and of the total mixture.

Treatment	Toxic contribution from salinity (TU)	Total TU atrazine/salinity	Total TU molinate/salinity	Total TU chlorpyrifos/salinity
Control	0.0	0.00	0.00	0.00
1	0.0	0.93	0.89	0.86
2	0.18	1.11	1.06	1.03
3	0.36	1.28	1.24	1.21
4	0.71	1.64	1.60	1.57
5	1.07	1.99	1.95	1.92
6	1.42	2.35	2.31	2.28

3.3.4.3. Group 3 experiments

Group 3 experiments consisted of equitoxic mixtures of the individual pesticides (i.e., atrazine / molinate, atrazine / chlorpyrifos, chlorpyrifos / molinate, atrazine / molinate / chlorpyrifos). In this set of experiments, each treatment consisted of pesticide concentrations representing equally toxic contributions from each pesticide (termed equitoxic mixtures of the pesticides) (Table 3.2). Ideally the total TUs for each treatment of each mixture should be identical but small variations did occur. The TU values were based on measured concentration values of each pesticide in the mixtures (Table 3.2)

Table 3.2: The measured composition of each treatment of the equitoxic pesticide mixtures expressed as toxic units (TU).

Treatment	Total Toxic Units in each mixture			
	Atrazine/ molinate	Atrazine/ chlorpyrifos	Molinate/ chlorpyrifos	Atrazine/ molinate/ chlorpyrifos
Control	0.00	0.00	0.00	0.00
1	0.23	0.22	0.22	0.22
2	0.45	0.45	0.44	0.44
3	0.91	0.89	0.87	0.89
4	1.36	1.34	1.31	1.33
5	1.81	1.78	1.74	1.78

3.3.4.4. *Group 4 experiments*

In group 4 experiments, the toxicity of complex combinations of pesticides with salinity (i.e., atrazine / molinate / salinity, atrazine / chlorpyrifos / salinity, molinate / chlorpyrifos / salinity, atrazine / molinate / chlorpyrifos / salinity) to *P. subcapitata* was studied. This was carried out with fixed toxicity contributions of pesticides, i.e., the mixture totalling 1 TU, with increasing concentrations of salinity (Table 3.3). Ideally the total TUs for each treatment of each mixture should be identical but small variations did occur. The TU values were based on measured concentration values of each pesticide in the mixtures (Table 3.3).

Table 3.3: The measured composition of each treatment of the complex mixtures of pesticides and salinity expressed in terms of toxic units (TU) of the salinity and of the total mixture.

Treatment	Toxic contribution from salinity (TU)	Total TU atrazine/ molinate/ salinity	Total TU atrazine/ chlorpyrifos/ salinity	Total TU molinate/ chlorpyrifos/ salinity	Total TU atrazine/ molinate/ chlorpyrifos /salinity
Control	0.0	0.00	0.00	0.00	0.00
1	0.00	0.91	1.00	0.87	0.89
2	0.18	1.08	1.18	1.05	1.07
3	0.36	1.26	1.36	1.23	1.25
4	0.71	1.62	1.71	1.58	1.60
5	1.07	1.97	2.07	1.94	1.96

3.3.5. Test method

The 72 hour growth inhibition test is a static test that assesses the chronic toxicity of toxicants to the freshwater unicellular green alga *P. subcapitata*. The procedure followed the UTS/NSW DECC Centre for Ecotoxicology Manual (2005), which was adapted from the USEPA (1993) and Environment Canada (1992) documents. In brief, exponentially growing *P. subcapitata* was exposed to a series of concentrations of the toxicant or mixtures in a static system for 72 hours under defined conditions. The growth of the algae exposed to the treatments was compared with that of algae in the diluent control (liquid algal culture medium). The growth of the algae was determined by cell yield, which is the change in the number of algal cells over the duration of the test.

Each toxicity test consisted of a control and solvent control where appropriate, and either five or six concentrations of the toxicant, each with five replicates. In the tests with individual toxicants, equitoxic mixtures, and salinity with pesticide mixtures five toxicant treatments were used while for the individual pesticides and salinity mixtures six toxicant treatments were used. The test volume was 6 mL contained in 20 mL scintillation vials. In addition, two replicates each of a control blank and sample blank which consisted of diluent water (liquid algal culture medium) and test solution without algae were prepared. Of the five replicates of test solutions for each treatment, four were used for determining algal density at the end of the test, while the fifth replicate was used for measuring physico-chemical parameters (temperature, pH, salinity and dissolved oxygen) of the test solutions at the beginning and end of the test.

The volume of the scintillation vials was not large enough to permit quantification of the pesticides. Therefore, the surface area to volume ratio of the scintillation vials was calculated (which was 3.7/cm). Clear screw-capped bottles with a similar surface area to volume ratio had a proportionate volume of test solution (i.e., 25 mL) added. Six replicates from each of the lowest, intermediate and highest concentrations (a total of 18 bottles) of the test treatments were prepared using identical procedures to those described above. Three replicates for each concentration (9 bottles) were immediately

analysed for pesticide concentrations. The remainder were incubated under identical conditions as the algal toxicity test treatments for 72 hours and the pesticide concentrations were then determined using the methods described below.

Three hours before initiating a test, a four to seven day-old culture of exponentially growing algae was washed to remove the old culture medium. This was performed by centrifuging, decanting and resuspending the algal cells four times in 20 mL of new liquid algal culture medium. The density of the washed algal suspension (the inoculum) was determined and the volume required to achieve a final cell density of 1 x 10^4cells/mL once the algae was added to 6 mL of test solution was calculated. This volume of inoculum was added to each of the test solutions apart from the blank solutions. All solutions were incubated at 24±1°C for 72 hours on a shaker table under cool white light with a light intensity of 4000 lux at the surface of the solutions.

The measured endpoint for the test was inhibition in growth of *P. subcapitata,* expressed as the reduction in cell yield relative to that of the control after 72 hours' exposure to the test solutions. After 72 hours, the cell densities in all the test vessels were counted using an electronic particle counter (LiQuilaz-E20 with a liquid sampler LS-200 and a software package, APSS-View-21 CFR11). Cell yield and percent effect (reduction in cell yield) relative to that of the controls were calculated. The recommended statistical endpoint was the estimated concentration that exerts a 50% growth inhibition effect – the IC50 value.

A reference toxicant test using sea water was run concurrently with each set of toxicity tests using the same algal suspension as that for the toxicity tests. The reference toxicant tests were conducted using identical procedures to those of the algal toxicity test described above. Results of the reference toxicity tests were compared to a cumulative summary (Cusum) chart to assess the acceptability of each batch of test organisms. Cusum charts were established following the methods of the (Environment Canada 1990).

The algae toxicity tests were considered valid if the:

- average algal cell density in the test medium control after 72 hours was at least 16 x 10^4 cells/mL;
- coefficient of variation (CV = SD / mean) of cell density in the control replicates did not exceed 20%; and
- the IC50 value obtained for a reference toxicant test was within two standard deviations (±2 SD) of the mean IC50 values from the Cusum chart.

3.3.6. Evaluation of mixture toxicity

The toxicity of individual components in mixtures and the mixtures themselves were expressed as toxic units (TUs) using the method described by Brown (1968). Typically the TU is calculated using,

$$TU_i = \frac{C_i}{EC_{p_i}} \qquad (1)$$

where the subscript denotes the component 'i' of a mixture, while C_i is the aqueous concentration of component 'I' in a mixture and ECp_i is the aqueous concentration of the component acting individually, which will cause a given toxic effect (e.g. IC50, IC20 values). In the present study the term ECp_i was always the IC50 (inhibition) value for *P. subcapitata*. Thus TU the values of 1 for individual chemicals mean that they are present in a mixture at their IC50 value.

The Plackett and Hewlett (1952) mixture classification scheme has four types of toxic interactions (Table 3.4). These are simple similar (more usually referred to as concentration addition, CA), independent (also more usually referred to as response addition, RA), complex similar and dependent joint action. Mathematical equations have been developed to express only CA and RA.

Table 3.4: The four types of joint action for mixtures, developed by Plackett and Hewlett (1952).

	Similar Joint Action	**Dissimilar Joint Action**
Non-interactive	Simple similar (concentration addition, CA)	Independent (response addition, RA)
Interactive	Complex similar	Dependent

Theoretically the CA type of joint interaction should only occur when a mixture consists of chemicals with the same mechanism of action and the RA is the type of additive joint interaction that should apply when mixtures contain chemicals with different mechanisms of action. As the three pesticides and salinity all have different mechanisms of action, theoretically, they should be modelled using the RA approach. However, we used the CA approach, as a number of laboratory and field-based studies have shown that CA overestimated the effects and yielded slightly higher estimates of the toxicity of mixtures than RA when the chemicals had different mechanisms of action (Faust, Altenberger et al. 1994; Warne and Hawker 1995; Ross and Warne 1997; Backhaus, Altenburger et al. 2000; Backhaus, Altenburger et al. 2000; Deneer 2000; Dyer, White-Hull et al. 2000; Chevre, Loepfe et al. 2006; Junghans, Backhaus et al. 2006). It is important to use the precautionary approach but not to be over protective in developing water quality guidelines, hence the CA is the preferred model over the RA model in the present study as the latter model would underestimate the combined effects of mixtures (Junghans et al. 2006).

The results of the mixture toxicity experiments were plotted as illustrated in Figure 3.1. In this figure the concentration of the mixture that causes a certain percentage of inhibition expressed in terms of toxic units. The solid line is the additivity line which links the points 0 TU, 0% inhibition; 0.5 TU, 25% inhibition; 1 TU, 50% inhibition; 2 TU, 100% inhibition. As indicated in ECETOC (2001), a less deviation of less than 30% from expected additivity is considered as conforming to additivity and a deviation of more than 30% from additivity conforms either to antagonism or synergism. The interpretations of mixture toxicity in the present study will be based

on the above concept. The two dashed lines (Figure 3.1) indicate the 30% deviation from additivity and mixtures that lie to the right of the lower dashed line are classified as antagonistic, while those that lie to the left of the upper dashed line are classified as synergistic. Mixtures that conform to CA will lie in between the two dashed lines

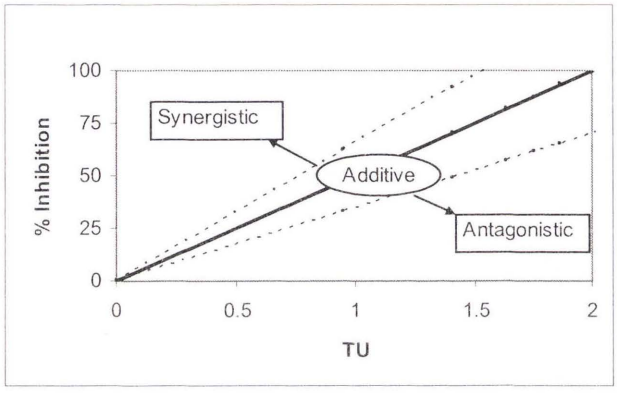

Figure 3.1: An example of the plots used to indicate the type of toxic interaction that occurs within the mixtures. The concentrations of the mixture (expressed as toxic units, TUs) that cause a certain % inhibition are plotted. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

3.3.7. Chemical analysis

The concentrations of the pesticides were determined using the NSW EPA screening and extraction method for volatile and semi-volatile organic compounds (NSWEPA 1998). Chemical analysis of test solutions was conducted at the beginning of each test and on completion, to determine the loss of pesticides during the test.

Extraction consisted of mixing a 25 mL aliquot from each treatment with 25 mL of dichloromethane in a 200 mL separatory funnel and shaking for two minutes with periodic venting. Two sequential extractions were conducted for each sample, and the extracts for each sample were combined. Each combined extract was dewatered by passing it through a 5 g bed of high-purity anhydrous Na_2SO_4. The dewatered extracts were concentrated down to 1 mL by evaporation using nitrogen gas under low

temperature (30°C). Solvent exchange was conducted using 4 mL acetone prior to analysis.

Chemical analysis was conducted using a gas chromatograph (HP 5890 series II Plus, with HP 3365 Series II Chemstation software) with a nitrogen phosphorus detector. The capillary column used for the analysis had a length of 30 m, an internal diameter of 0.25 mm, and was coated with 0.25 μm thick DB5 stationary phase. Injector port and detector temperatures were 250 and 220°C, respectively. Nitrogen was used as the carrier gas. The temperature programme was set to have an initial column temperature of 100°C for 1 min, which was raised to 275°C at the rate of 4°C per minute and held for one minute. A 1-μL sample injection volume was used throughout the analyses. Calibration standards were prepared for each chemical from the stock solutions and injected after every 10 injections of the samples. The respective standard curves for each pesticide were prepared and sample concentrations were determined using the linear regression method.

3.3.8. Calculations and statistical analysis

The concentration that inhibited cell yield by 50% after 72 hours exposure (72 hour IC50 value) and 95% confidence limits were calculated using the US EPA Linear Interpolation method (Norberg-King 1998). If the pesticides incurred a loss of less than 20% over the 72-hours incubation period, the measured initial concentrations were used to calculate their toxicities. If the loss was greater than 20% over the incubation period, the geometric means of the initial and final measured concentrations were used for the calculations.

3.4. Results

The ranges of each physicochemical property measured in all treatments of all the toxicity tests were within the acceptable limits.

3.4.1. Reference toxicant tests

The salinity IC50 values were well within two standard deviations of the long-term mean IC50 value (Figure 3.2). Thus, all the algal toxicity tests are considered valid and the sensitivity of *P. subcapitata* was uniform throughout the study and also the seawater (used for salinity treatments) quality is uniform and its toxicity did not vary between tests. This permits comparison of the toxicity results conducted at different times.

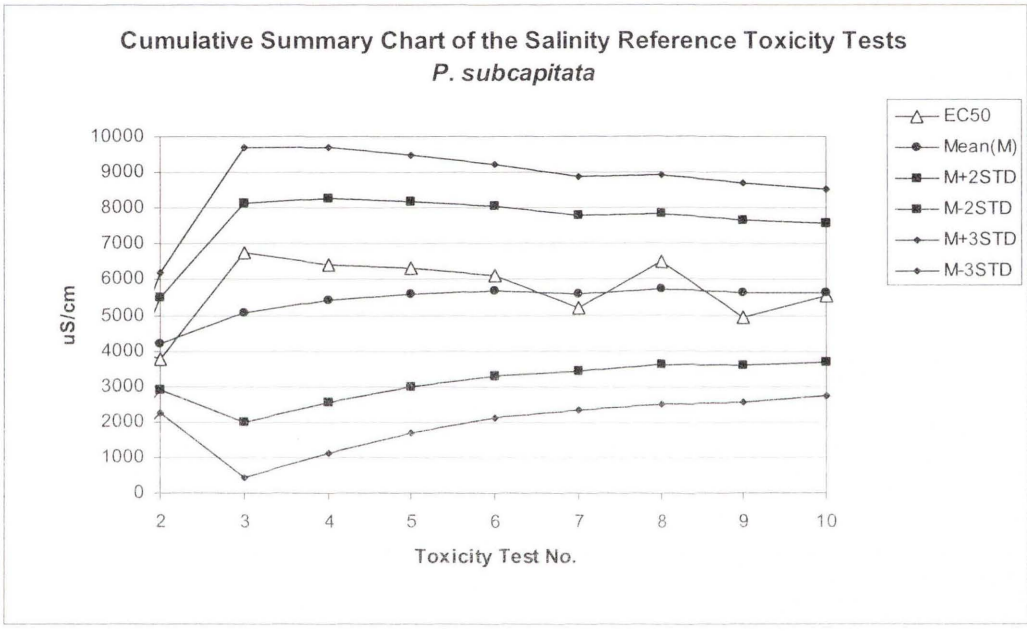

Figure 3.2: The cumulative summary chart for the salinity reference toxicity tests for *P. subcapitata*. (The actual IC50 values are represented by empty triangles, cumulative mean salinity is represented by filled circles, twice the standard deviations are represented by filled squares).

3.4.2. Toxicity experiments

3.3.2.1 Group 1 experiments

The chronic toxicities of salinity (mean), atrazine, molinate and chlorpyrifos (IC50 & IC25 values) are presented in Table 3.5. Based on the toxicant classification (USEPA 2006), the present results showed that atrazine was very highly toxic and molinate and chlorpyrifos were highly toxic.

Table 3.5: IC50 and IC25 values of salinity, atrazine, molinate and chlorpyrifos to *P. subcapitata* and their corresponding 95% confidence limits. The IC50 and IC25 results for salinity are the mean and ± standard error of ten values.

Toxicant	IC50	IC25
Salinity (μS/cm)	5598 ± 972	2948 ±123
Atrazine (μg/L)	47.8 (27.13 – 63.46)	19.9 (16.8 – 45.8)
Molinate (μg/L)	300.5 (262.1 – 316.4)	238.1 (168.9 – 258.6)
Chlorpyrifos (μg/L)	796.9 (672.9 – 1128.2)	528.9 (402.2 – 651.4)

To compare the toxicities of individual chemicals (Warne and Schifko 1999), the IC50 values were expressed in moles/L (Table 3.6). Of the two herbicides, atrazine was more toxic than molinate to the alga. The toxicity of the insecticide chlorpyrifos was lowest.

Table 3.6: IC50 values of atrazine, molinate and chlorpyrifos to *P. subcapitata* expressed in moles/L.

Toxicant	IC50 (moles/L)
Atrazine	2.22×10^{-7}
Molinate	1.60×10^{-6}
Chlorpyrifos	2.27×10^{-6}

Representative concentration response curves for each of the pesticides and salinity are illustrated in Figure 3.3. They all have typical sigmoidal and monotonic relationships.

The toxicities of the various mixtures are graphically illustrated in Figures 3.4 –3.6 and the IC50 values are tabulated in Tables 3.6 to 3.9.

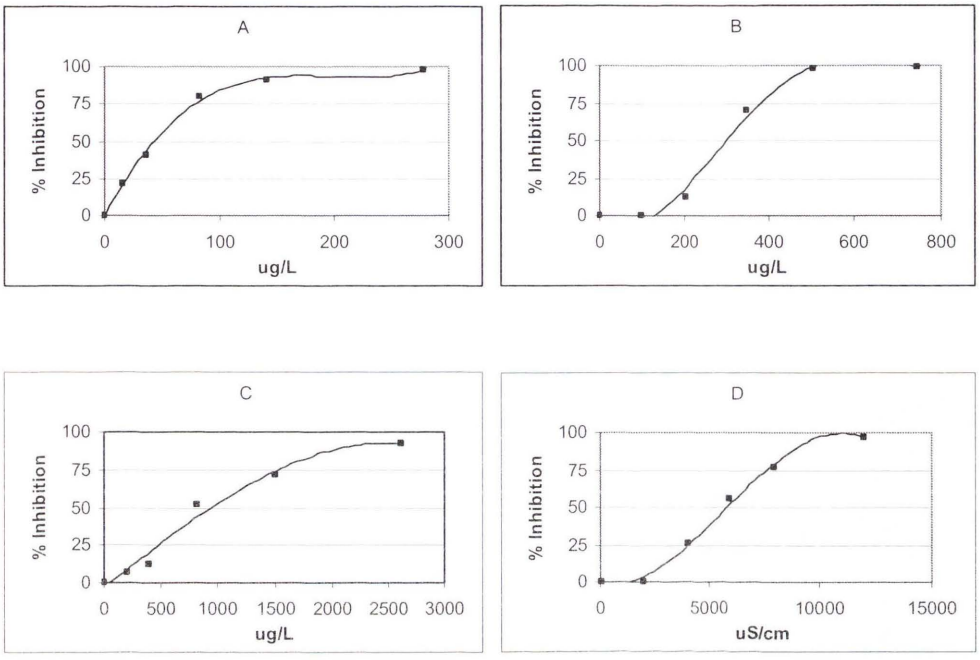

Figure 3.3: Typical concentration response relationships for the toxicities of individual pesticides (A - Atrazine, B - Molinate, C - Chlorpyrifos, and D - Salinity) to *P. subcapitata.*

3.4.2.2. Group 2 experiments.

The mixture of atrazine/salinity was additive while Treatment 1 (atrazine only treatment) was slightly over the 1 TU (Figure 3.4A). The toxicity relationship of molinate/salinity was synergistic in Treatment 2 but was additive for the remaining treatments (Figure 3.4B). The toxicity of the mixture of chlorpyrifos/salinity was antagonistic except for Treatments 1 and 6, which were additive (Figure 3.4C).

Since more than 50% inhibition was achieved in Treatment 1 in the atrazine/salinity and molinate/salinity mixtures it was not possible to calculate IC50 values based on toxic units (Table 3.7). The IC50 value for the chlorpyrifos/salinity mixture was 2.02 TU and conformed to antagonism (Table 3.7).

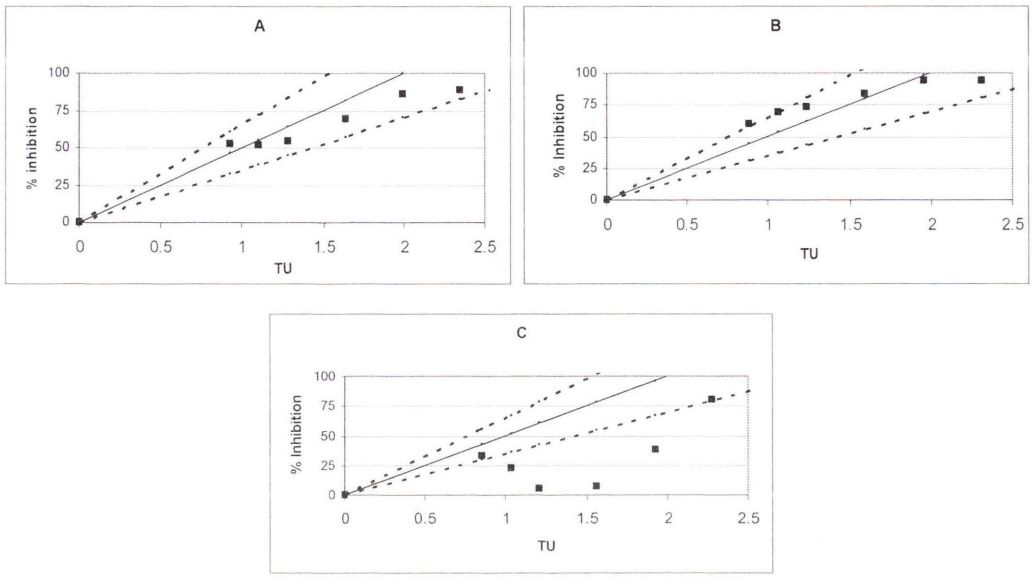

Figure 3.4: The toxicity of mixtures of individual pesticides with varying salinities to *P. subcapitata* cultured in normal algal media with a salinity of 100 µS/cm. A - Atrazine/salinity; B - Molinate/salinity; C - Chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

Table 3.7: The IC50 and 95% confidence limit values, expressed in toxic units (TUs), for the mixtures of individual pesticides (each present at a fixed concentration) and salinity.

Toxic Combinations	IC50 TU
Atrazine and salinity	< 0.93
Molinate and salinity	< 0.89
Chlorpyrifos and salinity	2.02 (1.89 – 2.09)

3.4.2.3. Group 3 experiments.

The variation in toxicity of the equitoxic pesticide mixtures with salinity is presented in Figure 3.5. The toxicity of the atrazine/molinate mixture was additive in Treatment 1, antagonistic in Treatments 2 and 3 and additive in Treatments 4 and 5 (Figure 3.5A). The toxicity relationship of atrazine/chlorpyrifos mixture was antagonistic except for Treatment 1 which was additive (Figure 3.5B). The toxicity relationship of the molinate/chlorpyrifos mixture was antagonistic (Figure 3.5C). The mixture of all three pesticides was additive in Treatment 1 and antagonistic in Treatments 2 and 3 additive in Treatments 4 and 5 (Figure 3.5D).

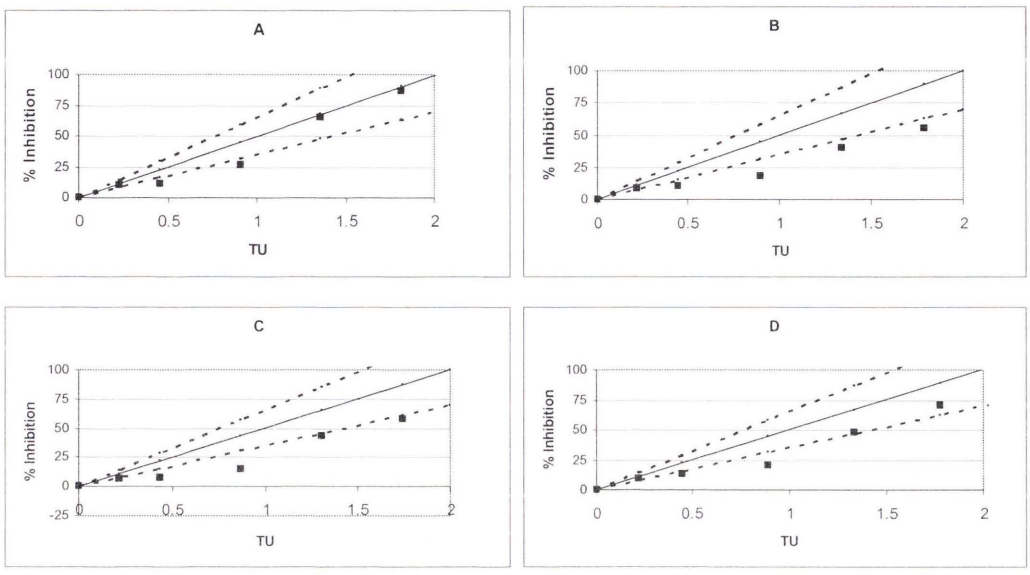

Figure 3.5: The toxicity of equitoxic mixtures of pesticides to *P. subcapitata* cultured in normal algal media with salinity of 100 µS/cm. A – Atrazine/molinate; B – Atrazine/chlorpyrifos; C – Molinate/chlorpyrifos, D - Atrazine/molinate/chlorpyrifos. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

Table 3.8 shows the IC50 values for each equitoxic combination of pesticides. Even though the toxicity relationship changed along the concentration gradients the toxicity relationships at the IC50 conformed to antagonism with the exception of the atrazine/molinate mixture.

Table 3.8: The IC50 and 95% confidence limit (95% CL) values of the equitoxic pesticide mixtures, expressed in toxic units (TUs)

Toxic Combinations	IC50 and 95% CLs (TU)
Atrazine and molinate	1.19 (1.08 – 1.35)
Atrazine and chlorpyrifos	1.63 (1.45 – 1.74)
Molinate and chlorpyrifos	1.49 (1.25 – 1.66)
Atrazine, molinate, chlorpyrifos	1.36 (1.27 – 1.46)

3.4.2.2. Group 4 experiments.

The variations in toxicities of complex mixtures of pesticides and salinity are presented in Figure 3.6. The toxicity relationship was additive for the atrazine / molinate / salinity mixture. A similar trend was found in the atrazine / chlorpyrifos / salinity mixture except for Treatments 1 and 3, which were marginally antagonistic (Figure 3.6B). The molinate/chlorpyrifos/salinity mixture conformed to antagonism (Figure 3.6C). The atrazine / molinate / chlorpyrifos / salinity mixture was antagonistic in Treatments 1 and 2, and additive in Treatments 3, 4 and 5 (Figure 3.6D)

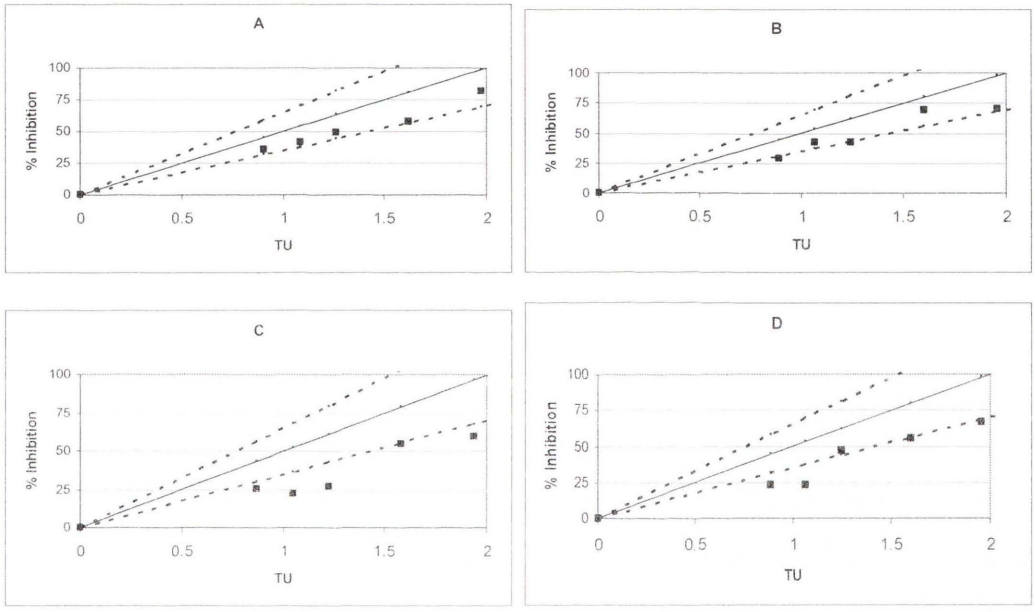

Figure 3.6: The toxicity of mixtures of pesticides with varying concentrations of salinities to *P. subcapitata* cultured in normal algal media with salinity of 100 µS/cm (the toxic contribution from the combination of pesticides is equal to 1TU). A - Atrazine/molinate/salinity; B - Atrazine/chlorpyrifos/salinity; C - Molinate/chlorpyrifos/salinity; D - Atrazine/molinate/chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

The IC50 values for the toxicity relationships for pesticide mixtures and salinity are presented in Table 3.9. The atrazine/molinate/salinity mixture was marginally additive while the rest of the pesticide mixtures were antagonistic.

Table 3.9: The IC50 values and 95% confidence limit (95% CL) values of the mixtures of pesticides with salinity, expressed as toxic units (TUs)

Toxicant Combinations	IC50 and 95% CLs (TU)
Atrazine, molinate and salinity	1.29 (0.99 – 1.64)
Atrazine, chlorpyrifos and salinity	1.43 (1.02– 1.59)
Molinate, chlorpyrifos and salinity	1.51 (1.39 – 1.89)
Atrazine, molinate, chlorpyrifos and salinity	1.38 (1.21– 1.69)

3.4.2.5 Occurrence of toxicity relationships

The types and the frequency of occurrence of toxicity relationships of the different mixture combinations studied are presented in Table 3.10.

Table 3.10: The types of toxic relationships observed in the mixture combination and frequency of occurrence.

Toxicity relationship	Frequency of occurrence
Additive	2
Antagonistic	2
Changed from antagonism to additivity*	1
Changed from synergism to additivity*	1
Changed from additivity to antagonism and back to additivity*	2
Changed from additivity to antagonism*	1
Changed from antagonism to additivity and then to antagonism*	1
Changed from antagonism to additivity, to antagonism and to additivity*	1
Total	11

* - Changes in toxicity relationship occurred with an increase in concentrations of mixtures.

Each mixture combination showed a variety of relationships. The majority of the mixtures was additive at least at higher concentrations (Table 3.10).

There were 55 different combinations of mixtures in the total of 11 mixtures studied. Table 3.11 summarises the number of types of toxicity relationships in these combinations. Approximately 51, 47 and 2% of these 55 mixtures conformed to antagonism, additivity and synergism, respectively.

Table 3.11: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos.

Toxicity relationship	Number of occurrence	% occurrence
Antagonistic	28	50.9
Additive	26	47.3
Synergistic	1	1.8

3.5. Discussion

Chronic exposure to the three pesticides and salinity inhibited the growth of *P. subcapitata*. The herbicide atrazine was most toxic; it inhibits photosynthesis by blocking the electron transport in the Photo-system II, which subsequently destroys the chloroplast, inhibits carbohydrate synthesis, which reduces the carbon pool and builds up CO_2 within the cell (Solomon et al. 1996). The IC50 value of atrazine to *P. subcapitata* in the present study which was 47.8 µg/L (2.22×10^{7} moles/L) was very similar to that observed by (Weiner et al. 2004), for *P. subcapitata* (96 hour IC50 as population growth rate) at 48.77 µg/L. Phyu (2004) reported the 72 hour IC50 value to *P. subcapitata* (growth inhibition) to range between 36.5 and 53.9 µg/L which concurs with the results of the present study.

Molinate which inhibits cell division in plants was second most toxic with an IC50 value of 300.5 µg/L (1.60×10^{-6} moles/L). Phyu (2004) recorded 72 hour IC50 values for *P. subcapitata* ranging between 55.3 and 115.6 µg/L, which were lower than the IC50 value found in the present study. The difference in toxicity of two herbicides atrazine and molinate could be due to differences in their modes of action.

Chlorpyrifos was less toxic compared with the two herbicides with an IC50 value of 796.9 µg/L (2.27×10^{-6} moles/L). This finding is to be expected, since plants do not contain the target site of chlorpyrifos to act on (i.e., the enzyme acetylcholine esterase). A LOEC value (72 hours test growth inhibition as endpoint) of chlorpyrifos to *P. subcapitata* of > 320 µg/L was reported by (Van Donk et al. 1992), which was lower than the result of the present study.

The mean IC50 value of salinity to *P. subcapitata* was 5598 µS/cm. Salinity affects osmoregulation in algae and changes the permeability of their plasma membranes (Reed 1984). The increased osmotic pressure in algal cells can inhibit photosynthesis thereby affecting their growth (Allakhverdiev et al. 2000). In addition, elevated salinity can depress the growth of algae through the toxicity of individual ions in the medium. Monovalant to divalent ion ratio is important in determining the toxicity of individual ions to algae (Cleave et al. 1981). In the present study, sea water was used to simulate various salinity treatments, since the composition of cations and anions in Australian inland salinity is comparable with that of sea water (Bayly and Williams 1972). Other sources of elevated salinity such as mining effluent can have higher divalent ion concentrations and therefore could be more toxic to aquatic organisms than sea water. As shown in the Cusum chart (Figure 3.2), the salinity toxicity to *P. subcapitata* remained uniform throughout the study, which indicates stable sensitivity of the test species.

The toxicity of the atrazine/salinity mixture was additive and that of the molinate/salinity mixture was also additive except for Treatment 2, which was synergistic. It is therefore evident that the combination of salinity and the two herbicides (individually) behaved according to the CA model. The antagonistic toxicity of chlorpyrifos and salinity could be due to changes in osmoregulation of *P. subcapitata* as a response to increased salinity, which could prevent the chlorpyrifos from entering the cells. The change in toxicity to conforming to additivity at high salinities could be due to salinity exerting either a direct toxic effect or increasing membrane permeability.

The equitoxic mixture of atrazine/molinate was additive in concentrations above 1 TU. The less than additive relationship below 1 TU could be due to each herbicide acting independently, and the concentrations not being sufficient to cause toxic effects at those concentrations, which is further supported by the IC25 values (Table 3.5) of each toxicant.

Mixtures that contained chlorpyrifos were predominantly antagonistic (Tables 3.7, 3.8 and 3.9). In field experiments, algal blooms were observed at low concentrations of

chlorpyrifos (Butcher et al. 1977; Van Donk et al. 1992). The most likely explanation for this was the decline in grazing pressure by herbivorous zooplankton. Butcher et al. (1977) also argued that the possible contribution of phosphorus from chlorpyrifos could be significant. However, there is no evidence of chlorpyrifos inducing growth of *P. subcapitata* at low concentrations in the present study (Figure 3.3C). In the chlorpyrifos/salinity experiment salinity-induced changes in osmoregulation seemed to be blocking chlorpyrifos from entering the algal cells, resulting in antagonistic toxicity. In the equitoxic experiments, the presence of chlorpyrifos could have decreased the effectiveness of the two herbicides; however the mechanism of such a phenomenon is still not clear.

While half of the mixtures tested were additive, they predominantly tended towards the lower edge of the envelope of additivity (i.e., towards antagonism). Mixtures comprising chemicals with the same mechanism of action (MeOA) should theoretically have a joint toxicity that conforms strictly to concentration addition (Deneer 2000; Faust et al. 1994; Warne and Hawker 1995) while those that have different mechanisms of action should conform to response addition (Table 3.4). A series of papers by Faust et al. (1994); Backhaus et al. (2000a and b), Dyer et al. (2000), Junghans et al. (2006) and Chevre et al. (2006) reported that concentration-addition overestimated effects and yielded slightly higher estimates of the toxicity of mixtures than response addition when chemicals had different MeOAs. These findings are also consistent with this theory (Plackett and Hewlett 1952). The mixtures studied had toxicities at the lower range of concentration addition, which was consistent with their joint toxicity conforming to response addition. This would be expected based on the fact that the components of the mixtures have different mechanisms of action.

The toxicities of 11 mixtures to *P. subcapitata* were determined in the present study (Table 3.10), giving a total of 55 combinations of mixtures. Of these, 51% of combinations conformed to antagonism, 47% conformed to additivity and 2% conformed to synergism (Table 3.11 Deneer (2000), Faust et al. (1994), Warne and Hawker (1995) and Ross and Warne (1997) found that approximately 5 – 15% of mixtures (irrespective of the type of chemical) were antagonistic. However, results of

the present study indicate that the ratio between additivity and antagonism was approximately 1:1 and synergism occurred in only one treatment.

The antagonistic toxicity relationships observed in the present study concurs with that of the review (Gressel 1990) which summarised information on the combined effects of herbicides on weed control, which indicated that there were far more reports on antagonism of herbicides than synergism. Even though there was no reference to atrazine and molinate in that review, it highlighted the fact that antagonism can occur in various combinations of herbicides.

3.6. Conclusions

Atrazine exerted a very high chronic toxicity to the alga, *P. subcapitata* (IC50 = 47.8 µg/L) while molinate and chlorpyrifos exerted a high chronic toxicity (IC50 = 300 and 800 µg/L, respectively).

Additive and antagonistic toxicity relationships observed in the present study occurred in similar proportions. Synergism was observed in only one combination. Based on the concentration addition model tested in the present study with *P. subcapitata*, sufficient protection for the freshwater algal species from the atrazine, molinate and chlorpyrifos mixtures in combination with salinity can be achieved if the additivity of the tested toxicants were taken into consideration in the derivation of water quality guidelines.

Chapter 4

Chronic toxicity of salinity and the pesticides, atrazine, molinate and chlorpyrifos individually and as mixtures to *Pseudokirchneriella subcapitata* acclimatised at elevated salinity

4.1. Abstract

Freshwater algae play an important role as primary producers in food chains in aquatic ecosystems. Algae inhabiting salinised inland water bodies are exposed to stress caused by salinity over generations and as a result, their sensitivity to other toxicants could be changed. Changes in sensitivity associated with such exposures have not been sufficiently studied. Salinity and pesticide pollution usually co-occur in the agricultural areas especially irrigated agriculture. Studies on the combined effects of salinity and pesticides on freshwater green algae are very limited, and particularly on these interactions when the algae are acclimatised to high salinities.

Cultures of the freshwater alga, *Pseudokirchneriella subcapitata,* were maintained in algal media of two salinities (i.e., normal salinity = 100 and high salinity = 6000 µS/cm) over five successive 72 hour long cultures (or approximately twenty cell doublings). Algal toxicity tests were conducted using these two cultures in order to determine the effects of long-term exposure to salinity on the toxicity of atrazine, molinate and chlorpyrifos and salinity individually, in mixtures of individual pesticides and salinity, in mixtures of several pesticides and salinity, and equitoxic mixtures of the pesticides. The concentrations that caused a 50% reduction in cell yield (IC50 values) for *P. subcapitata* cultured at high salinity were 5200 µS/cm, 43 µg/L, 282 µg/L and 1118 µg/L for salinity, atrazine, molinate and chlorpyrifos, respectively. Seventy-one percent of the mixtures tested conformed to additivity with 20% and 9% conforming to antagonism and synergism, respectively. For 13 out of 15 cases there were no significant differences in the sensitivity of the pesticides and their mixtures to the normal and high salinity acclimatised algal cultures. In both cases where differences occurred, the high salinity algal culture was more sensitive.

Acclimatisation to salinities up to 6000 µS/cm generally did not increase the risk posed by salinity and the three pesticides individually and in mixtures to *P. subcapitata*.

4.2. Introduction

Salinisation of landscapes is impacting on inland freshwater systems in some parts of the world (Williams 1999). Many freshwater systems in Australia are affected by salinity partly due to natural causes and more recently to human interventions such as clearing of deep rooted plants and irrigation activities (Jolly, Williamson et al. 2001; Nielsen, Brock et al. 2003). Jolly et al. (2001) reported a mean increase of 4.37 µS/cm/year with a minimum of -6.9 to a maximum of 139.5 µS/cm/year in streams in the Murrumbidgee Irrigation Area, NSW. Freshwater algae inhabiting salinised water bodies have been exposed to high salinity over generations. Exposure to a toxicant can cause variety of effects on their sensitivity and defence mechanisms (Smital et al. 2000). The possible scenarios of exposure to elevated salinity over generations are that the algae could become more sensitive or develop tolerance or retain their original sensitivity to salt and chemicals such as pesticides. According to the "metabolic cost" hypothesis (Calow and Sibly 1990) development of tolerance will increase energy consumption resulting ultimately in adverse effects on reproduction and growth. Thus, algae that are more tolerant of salinity may have less energy available to respond to other toxicants. Tolerance to other toxicants could decrease if they have the same mechanism of action as salinity or the same detoxifying or excretion mechanism. Tolerance to other toxicants could remain unaffected if the shift in tolerance is metabolically cost-neutral or if the organism's means of detoxifying or excreting the chemicals is not the same as that for salinity.

The use of agrochemicals has detrimental impacts on non-targeted species inhabiting freshwater environments through either runoff or spray drift. The impact is aggravated when salinity and pesticide contamination occur together. This is especially significant in irrigated agricultural areas.

Freshwater green algae as primary producers play a vital role in food chains and are important for the viability of communities inhabiting freshwater ecosystems. However, little information on how salinity-affected freshwater algae behave when they are exposed to other toxicants is available. Prediction of the effects of salinity and pesticide mixtures is difficult since salinity is not a conventional toxicant and therefore there should be systematic studies in order to understand the impacts of such mixtures on aquatic organisms. This chapter aims to contribute to this knowledge gap by determining how salinity affects the toxicity of mixtures of pesticides to salinity acclimatised alga, *P. subcapitata*.

The objectives of the study were:

- To determine the toxicity of salinity, atrazine, molinate and chlorpyrifos acting individually to the alga *P. subcapitata* cultured at high salinity (i.e., 6000 µS/cm) and to compare the differences in responses to the alga cultured at normal salinity (i.e., 100 µS/cm, the salinity of the algal culture medium).
- To determine the relationships of mixtures of pesticides, salinity and individual pesticides and complex mixtures of pesticides and salinity to salinity-acclimatised *P. subcapitata* and to compare their sensitivities with that of algae cultured at normal salinity (i.e., 100 µS/cm).

4.3. Materials and Methods

4.3.1. Test species

The green alga, *Pseudokirchneriella subcapitata* Hindak, formerly known as *Selenastrum capricornutum*, is a unicellular, non-motile, crescent-shaped (40–60 μm^3) alga that belongs to the Family Chlorophyceae. The algal culture (American Type Culture Collection [ATCC No. 22662]) is maintained at the Centre for Ecotoxicology laboratory and was originally obtained from the CSIRO Centre for Advanced Analytical Chemistry, Lucas Heights, NSW. This species was selected as it: represents primary producers and as such acts as an important food source for

herbivorous consumers such as cladocerans; is easy to culture in the laboratory; is extensively used in ecotoxicology; clumping of cells or formation of chains rarely occurs, thus enabling accurate enumeration using an electronic particle counter; grows sufficiently rapidly to permit an accurate measurement of cell yield after 72 hours; and is moderately sensitive to toxic substances (Environment Canada, 1997).

4.3.2. Toxicants

The pesticides used in the study were atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1, 3, 5-triazine-2, 4-diamine; CAS No-1912-24-9), molinate (s-ethyl *N*, *N*-hexa methylene thiocarbamate; CAS No-2212-67-1) and chlorpyrifos (O,O-Diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CAS No. 2921-88-2). They were all reagent-grade technical chemicals (≥ 97% purity). These three pesticides were selected since they are among the most commonly used pesticides in Australian agriculture (Bowmer et al. 1998; Thomas et al. 1998). Stock and working solutions of pesticides were prepared in analytical-grade (99% purity) acetone as a carrier solvent. The stock solutions were stored in a freezer at - 4°C and all working stock solutions were made immediately prior to use.

Sea water collected from Cronulla, NSW, was used for all toxicity tests, that required elevated salinity since the ionic composition of Australian inland saline waters is similar with that of sea water (Bayly and Williams 1972).

4.3.3. Algal cultures

The culture of algae followed the methods specified in USEPA (1993) and Environment Canada (1997). Details of the culture methods are described in the Chapter 2. High salinity (6000 µS/cm) were achieved by mixing appropriate amounts of filtered (using a 0.22 µm filter), UV-sterilised and aerated seawater with the Milli-Q water prior to the addition of the nutrient solutions listed in Chapter 2. The concentration of 6000 µS/cm for the elevated salinity cultures was based on the results of the chronic toxicity tests. For the experiments to work the algal cultures should be acclimatised and be able to be maintained for the exeriments duration, it

was therefore decided not to exceed 60% inhibition of algal growth to ensure there would be sufficient viable algal cells to complete the tests. At the salinity level of 6000 µS/cm there was on average approximately a 55% inhibition of growth (based on the chronic test results conducted for the salinity reference toxicity tests).

A monthly check on the quality of *P. subcapitata* in the cultures was performed by microscopic examination. The cultures were examined in terms of cell morphology, colour, clumping, absence of bacteria or yeast and measuring the relative sensitivity to a reference toxicant (sea salt).

4.3.4. Experimental design

The toxicity experiments for *P. subcapitata* were conducted in four groups similar to that described in Chapter 3 using the algae cultured at elevated salinities.

4.3.4.1 Group 1 experiments

The first group of experiments consisted of chronic toxicity tests with individual toxicants i.e., salinity, atrazine, molinate and chlorpyrifos. Based on these results, test concentrations to be used for the mixture toxicity tests were determined.

4.3.4.2 Group 2 experiments

The second group of experiments determined the toxicity of mixtures of salinity with individual pesticides (i.e., salinity and atrazine, salinity and chlorpyrifos, and salinity and molinate). These mixtures were made using a fixed concentration of each pesticide (i.e., the EC50 which equals 1 TU) with variable concentrations of salinity. The concentrations of salinity and each pesticide in each treatment of each mixture are presented in Table 4.1. Ideally the total TUs for each treatment of each mixture should be identical but small variations did occur. The TU values were based on measured concentration values of each pesticide in the mixtures

Table 4.1: The measured composition of each treatment of the individual pesticide and salinity mixtures expressed as toxic units (TU) of the salinity and of the total mixture.

Treatment	Toxic contribution from salinity (TU)	Total TU atrazine and salinity	Total TU molinate and salinity	Total TU chlorpyrifos and salinity
Control	0.0	0.00	0.00	0.00
1	0.0	0.93	0.89	0.86
2	0.18	1.11	1.06	1.03
3	0.36	1.28	1.24	1.21
4	0.71	1.64	1.60	1.57
5	1.07	1.99	1.95	1.92
6	1.42	2.35	2.31	2.28

4.3.4.3 Group 3 experiments

The third group of experiments consisted of equitoxic mixtures of individual pesticides i.e., atrazine / molinate, atrazine / chlorpyrifos, chlorpyrifos / molinate, and atrazine / molinate / chlorpyrifos. In this set of experiments, each treatment consisted of pesticide concentrations representing equally toxic contributions from each pesticide (termed equitoxic mixtures of the pesticides) (Table 4.2). The TU values were based on measured concentration values of each pesticide in the mixtures (Table 4.2). The total TUs for each treatment of each mixture should be identical but small variations did occur as the TU values were based on measured concentration values of each pesticide in the mixtures.

Table 4.2: The measured composition of each treatment of the equitoxic pesticide mixtures expressed as toxic units (TUs)

Treatment	Atrazine/ molinate	Atrazine/ chlorpyrifos	Molinate/ chlorpyrifos	Atrazine/molinate /chlorpyrifos
Control	0.00	0.00	0.00	0.00
1	0.23	0.22	0.22	0.22
2	0.45	0.45	0.44	0.44
3	0.91	0.89	0.87	0.89
4	1.36	1.34	1.31	1.33
5	1.81	1.78	1.74	1.78

4.3.4.4 Group 4 experiments

The toxicity of complex combinations of pesticides with salinity to *P. subcapitata* was determined (i.e., atrazine / molinate / salinity, atrazine / chlorpyrifos / salinity, molinate / chlorpyrifos / salinity, and atrazine / molinate / chlorpyrifos / salinity). The

treatments in these mixtures had a fixed toxic contribution from the pesticides (i.e., 1 TU) but varying concentrations of salinity (Table 4.3). The total TUs for each treatment of each mixture should be identical but small variations did occur as the TU values were based on measured concentration values of each pesticide in the mixtures.

Table 4.3: The measured composition of each treatment of the complex mixtures of pesticides and salinity expressed as toxic units (TU) of the salinity and of the total mixture.

Treatment	Toxic contribution from salinity	Total TU atrazine/ molinate/ salinity	Total TU atrazine/ chlorpyrifos/ salinity	Total TU molinate/ chlorpyrifos/ salinity	Total TU atrazine/ molinate/ chlorpyrifos/ salinity
Control	0.0	0.00	0.00	0.00	0.00
1	0.00	0.91	1.00	0.87	0.89
2	0.18	1.08	1.18	1.05	1.07
3	0.36	1.26	1.36	1.23	1.25
4	0.71	1.62	1.71	1.58	1.60
5	1.07	1.97	2.07	1.94	1.96

4.3.5. Test method

This 72 hour growth inhibition test assesses the chronic toxicity of toxicants to *P. subcapitata*. Inhibition in growth of the alga was the endpoint. This procedure was adapted from the USEPA (1993) and Environment Canada (1997) documents. Details of the test method are given in Chapter 3. *P. subcapitata* cultures acclimatised to high salinity (6000 µS/cm) over five successive 72 hr cultures were used instead of the alga cultured at normal salinity (100 µS/cm). The protocol for preparing the algal culture medium is described in Chapter 2. The high salinity algal medium (for the culture and for toxicity test treatments) was prepared by diluting filtered, aerated and UV sterilized sea water with polished deionised water before adding nutrient solutions (see Chapter 2).

A reference toxicant test using sea water was run concurrently with each set of toxicity tests on the same algal suspension used for the toxicity tests. The toxicity test procedure was identical to that of the test described in Chapter 3.

The measured endpoint for the test (concentration of the toxicant that causes 50% inhibition in the growth – IC50 value) was expressed as a reduction in cell yield relative to that of the control after 72 hours exposure to the test solutions. Algal growth inhibition was also expressed as a percentage effect relative to that of the control.

Toxicity tests were considered valid if:

- The average algal cell density in the control after 72 hours was at least 16 x 10^4 cells/mL;
- The coefficient of variation (CV) of cell density in the control replicates did not exceed 20%; and
- The IC50 value of the reference toxicant test was within twice standard deviations (±2 SD) of the mean IC50 values from the Cusum chart.

4.3.6. Evaluation of mixture toxicity - Toxic Units

The toxicity of individual components in mixtures and the mixtures themselves were expressed as toxic units (TUs) using the method described by Brown (1968). Typically the TU is calculated as follows,

$$TU_i = \frac{C_i}{EC_{p_i}} \qquad (1)$$

where the subscript denotes the component 'i' of a mixture, while C_i is the aqueous concentration of component 'i' in a mixture and ECp_i is the aqueous concentration of the component acting individually, which will cause a given toxic effect (e.g., IC50, IC20 values). In the present study the term ECp_i was always the IC50 (inhibition) value for *P. subcapitata*. Thus TU values of 1 for individual chemicals mean that they are present in a mixture at their IC50 values.

The results of the mixture toxicity experiments were plotted as illustrated in Figure 4.1. In this figure the concentration of the mixture that causes a certain % of inhibition is expressed in terms of toxic units. The solid line is the additivity line which links the points 0 TU, 0% inhibition; 0.5 TU, 25% inhibition; 1 TU, 50% inhibition; 2 TU, 100% inhibition. As indicated in ECETOC (2001), a less than 30% deviation from

expected additivity is considered as conforming to additivity and more than 30% deviation conforming either to antagonism or synergism. The interpretations of mixture toxicity in the present study will be based on the above concept. The two dashed lines (Figure 4.1) indicate the 30% deviation from additivity and mixtures that lie to the right of the lower dashed line are classified as antagonistic, while those that lie to the left of the upper dashed line are classified as synergistic. Mixtures that conform to CA will lie in between the two dashed lines.

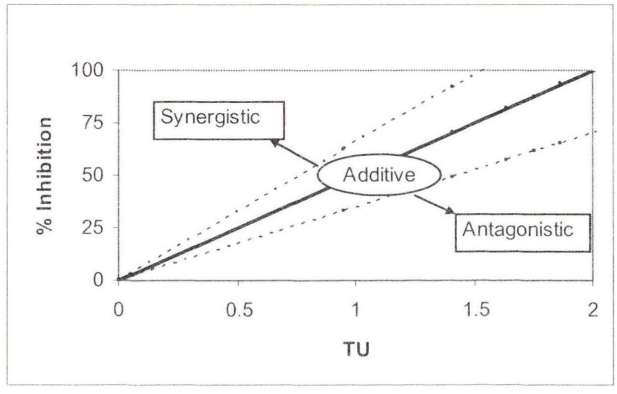

Figure 4.1: An example of the plots used to indicate the type of toxic interaction that occurs within the mixtures. The concentrations of the mixture (expressed in toxic units, TUs) that cause certain % inhibition are plotted. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

4.3.7. Chemical analysis

The concentrations of the pesticides were determined using the NSW EPA screening and extraction method for volatile and semi-volatile organic compounds. Chemical analysis of test solutions was conducted at the beginning and on completion of the test to determine the loss of pesticides during each test. Details of the chemical analysis procedure are described in Chapter 3.

4.3.8. Calculations and statistical analysis

The concentration that caused an inhibition of cell yield by 50% after 72 hours exposure (72 hour IC50) and 95% confidence limits were calculated using the US

EPA Linear Interpolation method (Norberg-King 1998). If the decrease in pesticide concentration was less than 20% over the 72 hours incubation period, the measured initial concentrations were used to calculate toxicity. If the decrease was greater than 20% during the incubation period, the geometric means of the initial and final measured concentrations were used for the calculations.

Statistical comparisons of IC50 values of the corresponding high-salinity-acclimatised and normal alga were conducted using the standard error of the difference test (Sprague and Fogels 1977).

4.4. Results

All the physico-chemical parameters for all the treatements in all the experiments were within the acceptable limits.

4.4.1. Reference toxicant tests

Figure 4.2 shows the variations in IC50 values for the reference toxicant (i.e., salinity). All values were within two standard deviations of the long-term mean and therefore the sensitivity of *P. subcapitata* was uniform through out the study and the results of toxicity tests could be compared. These results also indicate that the sea water (used for salinity treatments) quality is uniform and its toxicity did not vary between tests.

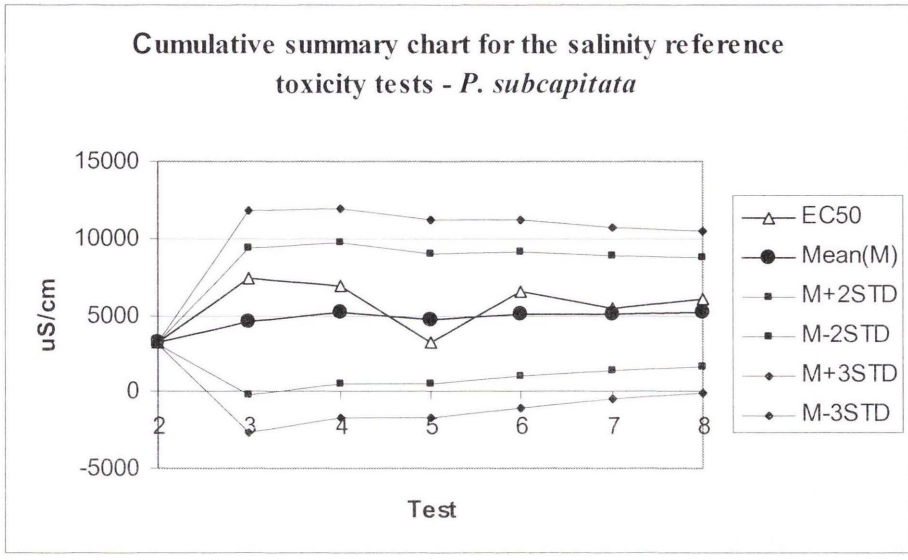

Figure 4.2: The cumulative summary chart for the salinity reference toxicity tests for *P. subcapitata* cultured at the salinity of 6000 µS/cm (The actual IC50 value is represented by empty triangles, cumulative mean salinity is represented by filled circles, twice the standard deviation is represented by filled squares).

4.4.2. Toxicity experiments

4.4.2.1 Group 1 experiments

The chronic toxicities of salinity (mean), atrazine, molinate and chlorpyrifos to the alga *P. subcapitata* cultured in normal salinity (100 µS/cm) and at high salinity (6000 µS/cm) are presented in Table 4.4. There were no significant differences ($p > 0.05$) between the IC50 values for each toxicant measured at the two salinities.

Table 4.4: Concentrations of salinity, atrazine, molinate and chlorpyrifos that caused a fifty percent reduction in cell yield (IC50) to *P. subcapitata* acclimatised and non-acclimatised to high salinity (6000 µS/cm) and the corresponding 95% confidence limits.

Toxicant	IC50 (cultured at 100 µS/cm)	IC50 (cultured at 6000 µS/cm)
Salinity* (µS/cm)	5598 ± 972	5201 ± 1759
Atrazine (µg/L)	47.8 (27.1 – 63.5)	42.7 (32.4 – 53.9)
Molinate (µg/L)	300.5 (262.1 – 316.4)	282.4 (240.03 – 311.9)
Chlorpyrifos (µg/L)	797 (673 – 1128)	1118 (900 – 1461)

* - mean IC50 of six salinity toxicity tests extracted from cusum chart.

Based on the USEPA toxicant classification (USEPA 2006), atrazine was very highly toxic, molinate was highly toxic and chlorpyrifos was moderately toxic (slightly over the limit of highly toxic). There were no statistically significant differences in IC50 values for the four toxicants between algae cultured at normal and at high salinity based on overlapping confidence limits.

To compare toxicities of individual chemicals in both the normal and high salinity cultures, the IC50 values were expressed in moles/L (Table 4.5) (Warne and Schifko 1999). The order of toxicity for high salinity acclimatised cultures was atrazine > molinate > chlorpyrifos and the order of toxicity was identical for the alga cultured at normal salinity.

Table 4.5: IC50 values of atrazine, molinate and chlorpyrifos to *P. subcapitata* expressed in moles/L.

Toxicant (moles/L)	IC50 (cultured at 100 µS/cm)	IC50 (cultured at 6000 µS/cm)
Atrazine	2.22×10^{-7}	1.98×10^{-7}
Molinate	1.60×10^{-6}	1.51×10^{-6}
Chlorpyrifos	2.27×10^{-6}	3.19×10^{-6}

The IC50 values for the six salinity reference toxicant tests conducted under normal and under high salinity conditions are presented in Table 4.6. Only in one instance was the IC50 value of the acclimatised culture significantly ($p \leq 0.05$) different to that of non-acclimatised culture of *P. subcapitata*. Thus, overall there was no difference in the toxicity of salinity.

Table 4.6: The IC50 values and 95% confidence limits (in parentheses) for the toxicity of salinity to *Pseudokirchneriella subcapitata* cultured in normal (i.e. 100 µS/cm) and high salinity (i.e., 6000 µS/cm); six reference tests.

IC50 values for *P. subcapitata* (µS/cm)	
Cultured at normal salinity (i.e. 100 µS/cm)	**Cultured at high salinity (i.e. 6000 µS/cm)**
3728 (3412 – 4377)	3148 (2069 – 6185)
4612* (3779 – 5845)	3174* (2701 – 3485)
6368 (4918 – 7079)	7339 (5970 – 8572)
6257 (5336 – 7135)	6852 (5975 – 8241)
6489 (5875 – 7026)	6480 (3488 – 6962)
5546 (3609 – 6520)	5371 (4627 – 6221)

* - Significantly different at ($p \leq 0.05$)

4.4.2.2 Group 2 experiments

The toxicity of the mixtures of salinity and each pesticide to the high salinity acclimated alga is presented in Figure 4.4. The toxicity of all treatments of atrazine / salinity and molinate / salinity mixtures conformed to concentration addition (additivity) except for Treatments 1, 4 and 5 of the chlorpyrifos / salinity mixture, which conformed to antagonism. These results were confirmed with the IC50 values for these mixtures (expressed in TUs) presented in Table 4.7. Statistical comparisons between IC50 values of *P. subcapitata* cultured in the normal and high salinity could only be made for the chlorpyrifos and salinity mixture. This mixture was significantly ($p \leq 0.05$) less antagonistic in the high salinity culture and/or the high salinity culture was significantly more sensitive than that in the normal salinity culture.

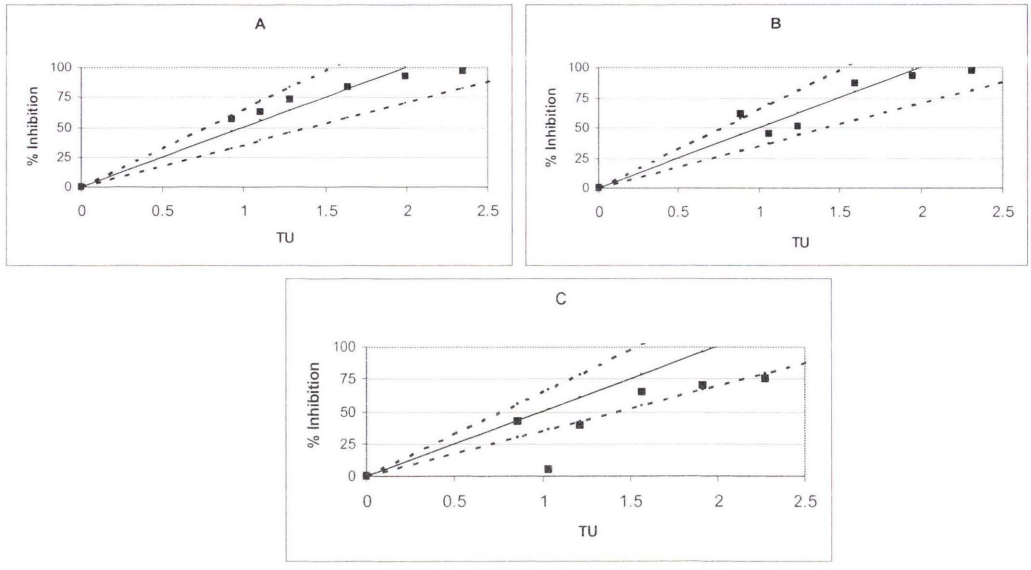

Figure 4.4: The toxicity of mixtures of individual pesticides with varying salinities to *P. subcapitata* cultured in high salinity algal media with a salinity of 6000 µS/cm. A - Atrazine/salinity; B - Molinate/salinity; C - Chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

Table 4.7: The IC50 and 95% confidence limit values, expressed as toxic units (TUs), for the mixtures of individual pesticides (each present at a fixed concentration) and salinity for *P. subcapitata* cultured in normal and high salinity algal media.

Toxic combinations	*P. subcapitata* IC50 values (TU)	
	Normal salinity culture (100 µS/cm)	High salinity culture (6000 µS/cm)
Atrazine IC50 and salinity	<0.93	<0.93
Molinate IC50 and salinity	<0.89	<0.89
Chlorpyrifos IC50 and salinity	2.02* (1.89 – 2.09)	1.36* (1.42 – 1.74)

* IC50 values are significantly ($p \leq 0.05$) different.

4.4.2.3 Group 3 experiments

The results of group 3 experiments i.e., toxicity relationships of the equitoxic mixtures of pesticides are presented in Figure 4.5. They conformed to additivity, except for Treatment 1 in the atrazine / molinate mixture, Treatment 2 in the atrazine / chlorpyrifos mixture and Treatment 1 in the atrazine / molinate / chlorpyrifos mixtures, where the relationships were synergistic. No significant differences ($p > 0.05$) were observed between the normal and high salinity cultures for equitoxic

mixtures. However, the IC50 values of the high salinity cultures were consistently lower than those of the normal salinity cultures (Table 4.8).

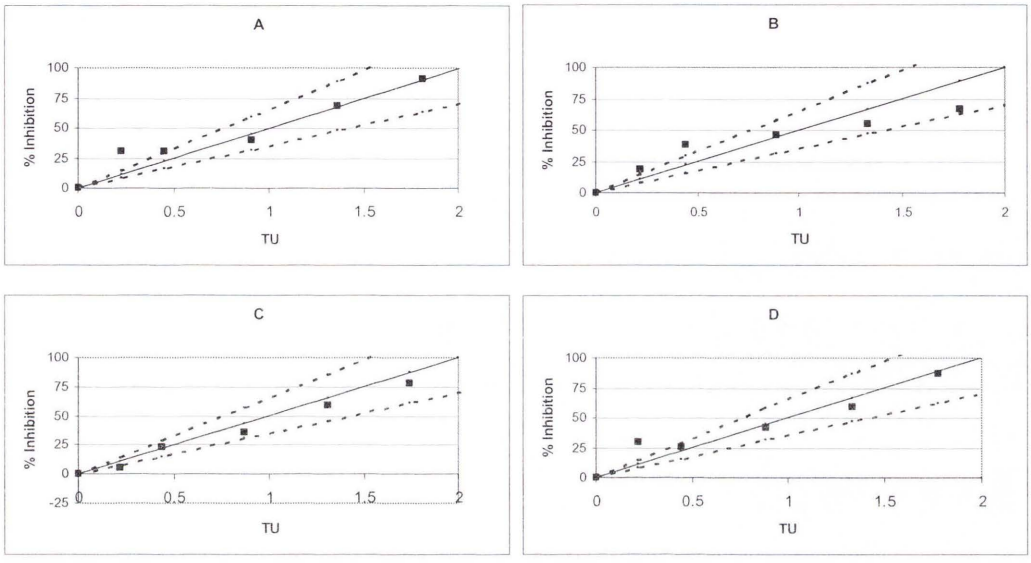

Figure 4.5: The toxicity of equitoxic mixtures of pesticides to *P. subcapitata* cultured in high salinity algal media with salinity of 6000 µS/cm. A – Atrazine/molinate; B – Atrazine/chlorpyrifos; C – Molinate/chlorpyrifos, D - Atrazine/molinate/chlorpyrifos. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

Table 4.8: The IC50 and 95% confidence limit (95% CL) values of the equitoxic pesticide mixtures, expressed as toxic units (TUs) for *P. subcapitata* cultured in normal and high salinity algal media.

Toxic Combinations	*P. subcapitata* IC50 values (TU)	
	Normal salinity culture (100 µS/cm)	High salinity culture (6000 µS/cm)
Atrazine and molinate	1.19 (1.08 – 1.35)	1.05 (0.68 – 1.3)
Atrazine and chlorpyrifos	1.63 (1.45 – 1.74)	1.15 (0.71 – 1.38)
Molinate and chlorpyrifos	1.49 (1.25 – 1.66)	1.14 (0.74 – 1.37)
Atrazine, molinate, and chlorpyrifos	1.36 (1.27 – 1.46)	1.09 (0.75 – 1.28)

4.4.2.4 Group 4 experiments

Figure 4.6 shows the variations in the toxicity of the complex pesticides and salinity mixtures with increasing salinity. With the exception of all treatments in the molinate / chlorpyrifos / salinity mixture and Treatments 2 and 5 of the atrazine / molinate / chlorpyrifos / salinity mixture, the remaining treatments of the complex mixtures conformed to additivity (Figure 4.6). The non-additive treatments conformed to antagonism.

The IC50 values for the high salinity cultures were significantly ($p \leq 0.05$) lower than their corresponding values for the normal salinity cultures for the atrazine / chlorpyrifos / salinity mixture (Table 4.9). The other mixtures had similar toxicity to the normal and high salinity cultures.

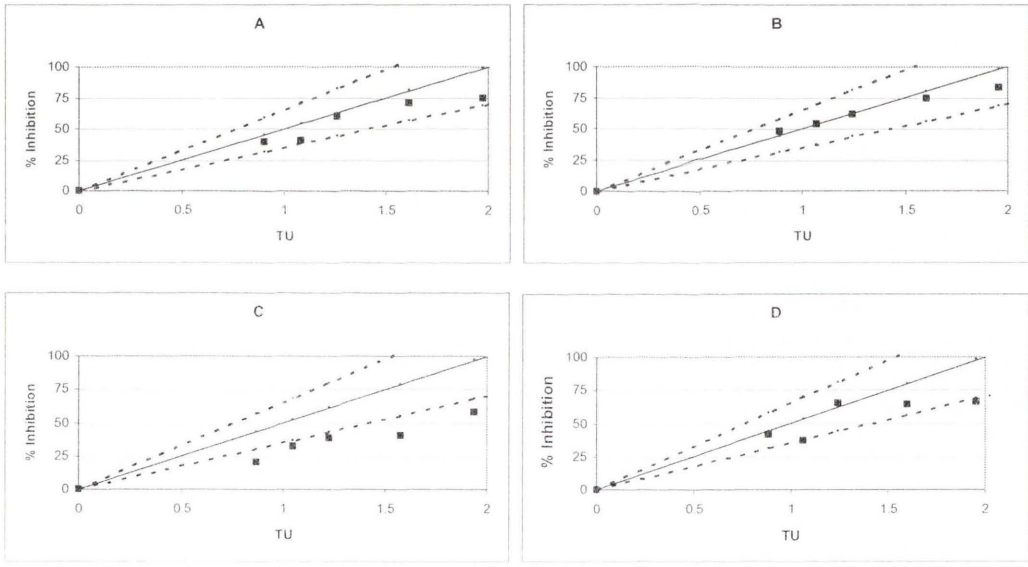

Figure 4.6: The toxicity of mixtures of pesticides with varying concentrations of salinities to *P. subcapitata* cultured in high salinity normal algal media with salinity of 6000 µS/cm (the toxic contribution from the combination of pesticides is equal to 1TU). A - Atrazine/molinate/salinity; B - Atrazine/chlorpyrifos/ salinity; C - Molinate/chlorpyrifos/salinity; D - Atrazine/molinate/chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. Each dot represents a treatment.

Table 4.9: The IC50 values and 95% confidence limit (95% CL) values of the mixtures of pesticides with salinity, expressed as TU for *P. subcapitata* cultured in normal and high salinity algal media.

Toxic Combinations	IC50 TU	
	Normal salinity culture (100 μS/cm)	High salinity culture (6000 μS/cm)
Atrazine, molinate and salinity	1.29 (0.99 – 1.64)	1.17 (1.10 – 1.25)
Atrazine, chlorpyrifos and salinity	1.43* (1.02– 1.59)	0.94* (0.90 – 1.28)
Molinate, chlorpyrifos and salinity	1.51 (1.39 – 1.89)	1.78 (1.22 – 1.89)
Atrazine, molinate, chlorpyrifos and salinity	1.38 (1.21– 1.69)	1.14 (1.11 – 1.22)

* - Significantly different at ($p \leq 0.05$)

4.4.2.5 Occurrence of toxicity relationships

The types and the frequency of occurrence of toxicity relationships for the different mixtures studied are presented in Table 4.10.

Table 4.10: The types of toxic relationships observed in the mixtures studied and their frequency of occurrence.

Toxicity relationship	Frequency of occurrence
Additive	4
Antagonistic	1
Changed from antagonism to additivity*	1
Changed from synergism to additivity*	3
Changed from additivity to antagonism, to additivity and to antagonism*	1
Changed from antagonism to additivity and then to antagonism*	1
Total	11

* - Changes in toxicity relationship occurred with an increase in concentrations of mixtures.

Each mixture combination showed a variety of relationships. The majority of the mixtures was additive, at least at the higher concentrations tested (Table 4.10).

There were 55 combinations of mixtures in the 11 mixtures studied. Table 4.11 summarises the number and percentage occurrence of the types of toxicity relationships in these mixtures. For the high salinity acclimated *P. subcapitata* culture approximately 20, 71 and 9% of the mixtures conformed to antagonism, additivity and synergism, respectively. Compared to the non-acclimated culture, acclimation

decreased the percentage that conformed to antagonism by 30% and this led to an identical increase in the percentage that conformed to additivity and synergyism.

Table 4.11: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos for *P. subcapitata* cultured at normal salinity and high salinity.

Toxicity relationship	*P. subcapitata* cultured at normal salinity		*P. subcapitata* cultured at high salinity	
	Number of occurrence	% occurrence	Number of occurrence	% occurrence
Antagonistic	28	50.9	11	20.0
Additive	26	47.3	39	70.9
Synergistic	1	1.8	5	9.1

4.5. Discussion

In this chapter comparisons were made between algal populations cultured at high salinity (6000 µS/cm) with that cultured at normal salinity (100 µS/cm) to determine if they responded similarly to pesticides and their mixtures, and increasing salinity.

A total of 11 mixtures had their toxicity to *P. subcapitata* determined in the present study (Table 4.11), giving a total of 55 combinations of mixtures. Of these 20% and 9% of the combinations of mixtures conformed to antagonism and synergism respectively while 71 % combinations conformed to additivity in the high salinity acclimated culture. These percentages concur with the ranges reported by Deneer (2000), Faust et al. (1994), Warne and Hawker (1995) and Ross and Warne (1997) who found that between 5 – 15% of mixtures (irrespective of the type of chemical) were antagonistic (20% in present study) and a similar percentage were synergistic (9% in present study).

By assuming concentration addition as the type of toxicant interaction for the mixtures examined in the present study, the toxicity of approximately 90% of the mixtures would be estimated accurately or overestimated. Thus, concentration addition is an appropriate model to use for determining the toxicity of these mixtures to *P. subcapitata*.

However, there are notable differences in percentage occurrence of different toxicity relationships between the high salinity acclimatised and normal salinity algal cultures. In high salinity cultures antagonistic relationships were reduced from 50.9% to 20%, additive relationships increased from 47.3% to 70.9% and synergistic relationships increased from 1.8% to 9.1% compared with normal cultures (Table 4.11). These findings indicate that *P. subcapitata* cultured at high salinity had increased sensitivity to pesticide and salinity mixtures to a certain degree. Possible reasons could be salinity induced osmotic stress and changes in membrane permeability (Reed 1984) and salinity effects on phosphate metabolism (Rai and Sharma 2006) in the algae. The effects of different metallic ions in salt may not have a significant role since sea water has less toxic metals compared with saline effluents such as mine effluent, which has more toxic divalent ions (Yim et al. 2006).

The results of the present study should be interpreted conservatively as only one elevated salinity treatment was used (i.e., 6000 µS/cm). If more salinities had been tested it would have been possible to discern whether there was an overall trend of increasing difference between the toxicity values generated for the normal and high salinity cultures. If that had occurred then it could be inferred that at some higher salinity, more significant ($p \leq 0.05$) differences in toxicity would occur.

Due to the experimental design used in the current study the results of the above comparisons could be due to the long-term exposure of *P. subcapitata* to the different salinities (i.e., the effects of acclimatising to the different salinities) or to the effects of exposure to the different salinities over 72 hours of the toxicity tests (i.e., the salinities having immediate effects on uptake, solubility and metabolism of the pesticides and salinity). This occurs because the *P. subcapitata* cultures acclimatised to the different salinities were tested in those same salinity media. This could have been overcome by culturing the algae in normal and high salinity media, as was done here, but then transferring them to normal salinity media for conducting the tests. However, this is also likely to create a problem - such a rapid change in salinity could cause toxic shock to the algae that would override the toxicity or at least contribute to the toxicity measured under these circumstances.

Some freshwater algae have been shown to exhibit physiological adaptations and homeostatic responses to the exposure to elevated salinity and hence they have shown less stress and health effects (Kirst 1977; Lorenzo et al. 2007). Some of the physiological adaptations include maintaining the osmotic potential by changing the sucrose concentration in vacuoles in response to salinity and also by changing the membrane permeability. These phenomina were observed in freshwater algae that belong to Characeae (Bisson and Kirst 1983). In the case *of Kirchneriella* sp., several researchers (Kirst 1977; Lorenzo et al. 2007) concluded that the alga acclimates to different salinity (NaCl) concentrations, at the chloroplast level. They postulated that the alga acclimated by altering the LHCII-PSII coupling and at high NaCl concentrations, changing the thylakoid (membrane-bound compartment inside chloroplasts) stucture and the PSII to allow sufficient integrity of the photosynthetic membrane. In addition they also stated that the mucilaginous capsule surrounding the alga might play an important role in regulating the ionic balance. Therefore it appears that at least some freshwater alga species can adapt to unfavourably high salinity levels without loosing their ability to survive or alter their response to other toxicants. The observations of the present study may be due to the above or similar physiological adaptations and homeostatic responses of the *P. subcapitata* to the elevated salinity.

Given the above, we can state that culturing *P. subcapitata* at 6000 µS/cm for five successive 72 hour long cultures (or approximately twenty cell doublings) and conducting toxicity tests did not alter their sensitivity in terms of IC50 values of individual and mixture pesticide toxicities (except for two mixtures). However, in terms of overall percentage occurrence of toxic relationships, antagonism decreased and additivity increased in high salinity cultures in response to toxicity of these mixtures.

4.6. Conclusions

With regards to individual toxicities of pesticides, atrazine was very highly toxic, molinate was highly toxic and chlorpyrifos was moderately toxic.

The results suggest that exposure to higher salinity (i.e., 6000 µS/cm) over five successive 72 hour long cultures (or approximately twenty cell doublings) did not affect the sensitivity of the *P. subcapitata* to the pesticides atrazine, chlorpyrifos, molinate and salinity individually. For mixtures of the individual pesticides and salinity, mixtures of several pesticides and salinity, and equitoxic mixtures of the pesticides, the majority of the concentrations (71%) tested conformed to concentration addition. Approximately 20% and 9% of the mixtures tested conformed to antagonism and synergism, respectively. In terms of IC50 values, about 70% of the mixtures did not show sensitivity changes. Changes in toxic relationships in mixtures occurred i.e., antagonistic mixtures in non-acclimatised cultures became additive in high salinity acclimatised cultures. It could therefore be inferred that there is an indication of certain degree of change in sensitivity at a salinity of 6000 µS/cm to *P. subcapitata*.

Chapter 5

Effects of multigenerational exposure to salinity on reproduction, growth, feeding and life history traits of *Daphnia carinata*

5.1. Abstract

The salinisation of freshwater bodies is a serious environmental issue in many parts of the world. It has created serious concerns about the impact on freshwater biota and on ecosystem functions. The present study determined the effects of salinity on the reproduction, growth, feeding and life history traits of *Daphnia carinata* when maintained over four generations (i.e., F0, F1, F2, and F3) in water at five salinities viz. 200 (normal cladoceran water), 2000, 4000, 5500 and 6300 µS/cm. The third brood neonates of each generation were used to commence the next generation. Daily observations were made, all neonates were counted and the mean total reproduction per female was estimated for each treatment in each generation. The intrinsic rate of natural increase was calculated using life tables for each generation in each treatment. The time to the first brood for each generation and the mean generation time in relation to the first three broods were also recorded. The lengths of the adults after producing the third brood of each generation (except F3) and the lengths of less than 24 hours old neonates from the second brood of the F0 – F2 generations were also recorded. The filtration and ingestion rates of the neonates aged between 24 and 48 hours of the F0, F1, and F2 generations were also determined.

The sensitivity of *D. carinata* to salinity increased over generations with regard to mean total reproduction per female and mean intrinsic rate of natural increase. Salinities greater than or equal to 5500 µS/cm significantly reduced ($p \leq 0.05$) the reproductive ability, adult lengths, intrinsic rate of natural increase and ingestion rate of *D. carinata*. Neonatal lengths and filtration rates were significantly reduced ($p \leq 0.05$) at a salinity of 2000 and 4000 µS/cm, respectively. In the F0 generation, slight increases in adult length, brood size and the intrinsic rate of natural increase were found at 2000 µS/cm, which could be due to less energy being needed for

osmoregulation at that salinity. Significant delays ($p \leq 0.05$) in the production of neonates (time to first brood and generation time) also occurred with increasing salinities.Development of tolerance to salinity as an adaptation over generations was not clearly evident from the results. The results however suggest that the salinisation of inland waters may have long-term impacts on the viability and survival of freshwater organisms.

5.2. Introduction

The salinisation of freshwater environments has been identified as a continuous process in many parts of the world (Jolly et al. 2001; Williams 1987). There is some information available on the effects of salinisation on the aquatic environment (Halse et al. 1998; Hart et al. 1991; James et al. 2003; Kefford 1998; Kefford et al. 2006; Kefford et al. 2005; Kefford et al. 2003). Increased salinity affects the ecological structure of the impacted environments by causing physiological stress in individual freshwater organisms. It has been emphasised that salinity acts as a toxicant when it exceeds certain threshold levels in freshwater organisms (Kefford et al. 2002).

The variations in salinity in different inland water bodies in Australia show quite a wide range (Pinder et al. 2005). Table 5.1 shows the range of salinities in inland water bodies in the Western Australian wheat belt.

Table 5.1: Salinities of water bodies in the Western Australian wheat belt (Source: Pinder et al. 2005)

No. of sites	Salinity µS/cm
86	<4300
46	4300 – 14000
69	14000 - 140000

Salinity-influenced areas are subject to fluctuations in salinity over a very long time and there are increasing trends of salinity in many areas. Jolly et al. (2001) reported the average rates of increase in salinity (corrected linear electrical conductivity trends) in streams of the Murray-Darling Basin at 87 monitoring stations (Table 5.2).

Table 5.2: Mean salinities and salinity trends in streams in Murrumbidgee Irrigation Area (Source: Jolly et al. 2001)

	Mean ± Standard Error)	Minimum	Maximum
Salinity (μS/cm)	442 ± 81	46	6573
Salinity trend (μS/cm/year)	4.37 ± 2.03	-6.9	139.5

To study long term-exposure scenarios, chronic and multigeneration studies are more appropriate than acute tests. Multigeneration studies are preferred over chronic studies, since they provide information on population-level effects over generations, which are more realistic for understanding the long-term viability of the species of concern. Long-term exposure to toxicants over generations has been studied for various classes of chemicals using a range of organisms. The effects of pesticides on generations pre-exposed to pesticides of *Daphnia* were discussed by Villarroel et al. (2000), while Rose et al. (2002a, 2002b) studied similar aspects of an organic solvent and pesticides. Long-term pesticide exposure studies have also been conducted by Ferrando et al. (1996), Vander Hoeven and Gerritsen (1997) and Villarroel et al. (2000a and b). Information is also available on inorganic chemicals (Radix et al. 2000; Vogt et al. 2007), organic chemicals (Tanaka and Nakanishi 2002). Studies on heavy metal acclimation over multiple generations were undertaken by (Bossuyt et al. 2005), Muyssen and Janssen (2002) and Tsui and Wang (2007). However, only a limited amount of information is available on the long-term toxicity of salinity on freshwater organisms (Kefford et al. 2004).

Zooplankton including *Daphnia* spp. have been used to study some aspects related to salinity effects. Arner and Koivisto (1993) worked on the metabolism and life history of *Daphnia magna,* while Teschner (1995) studied the life history and fitness of different clones of *D. magna* found in freshwater and brackish water habitats. The tolerance and potential use of *D. magna* for estuarine toxicity tests were studied by (Schuytema et al. 1997) while the scope for growth and fitness of *D. magna* was studied by Baillieul et al. (1996). The growth and mortality of *D. carinata* with salinity and temperature was studied by Hall and Burns (2002). Kefford et al. (2002) has presented the acute toxicities of different salts, including sea salt, to *D. carinata.* Information on osmotic regulation in crustaceans and cladocerans in particular

including osmoregulatory organs, membrane permeability, adaptations and haemolymph osmotic potential are available (Aladin and Potts 1995; Pequeux 1995). Information on salinity covering the whole range of toxic effects, viz. growth, reproduction, life history and feeding for one organism is very rare. It is very important to study all these aspects on appropriate test species that are found in a highly salinity-affected country like Australia.

To understand and evaluate the effects of salinity, it is very important to select species representing ecologically relevant groups of organisms. The use of a single test species would not provide information on the impact of salinity at the community level. Therefore, in addition to the fresh water alga *Pseudokirchneriella subcapitata* (see Chapter 2), the freshwater cladoceran *Daphnia carinata*, which is found in Australia, was selected to represent primary consumers in freshwater environments.

The aim of the present study was to assess the effects of multi-generational exposure to elevated salinity on reproduction, growth, feeding and life history traits of *Daphnia carinata*.

5.3. Materials and Methods

5.3.1. *D. carinata* cultures

D. carinata was cultured following the methods described in the USEPA (2002). *D. carinata* were originally obtained from the Fisheries Research Station at Narrandera, NSW (Julli, pers. comm.).

D. carinata cultures were maintained in 2L beakers which contained 1.8 L of cladoceran water and five adults. Cladoceran water is Sydney mains water that has been passed through a 1 μm filter, treated with sodium thiosulphate (to remove traces of residual chlorine) and aerated at 20°C for at least 24 hours before use. Cladoceran water was renewed three times a week, i.e., on Monday, Wednesday and Friday. The renewed cultures were fed two species of green algae, *Ankistrodesmus* sp. and

Pseudokirchneriella subcapitata (50,000 cells/mL of each algal species). The cultures were maintained in a 16: 8 hours light : dark cycle, at 20 ±1°C.

The salinity of Sydney main water after extended aeration ranged from 190 to 200 µS/cm. The recommended optimum salinity for culturing and conducting toxicity tests using *D. carinata* in the Methods Manual of the Ecotoxicology Section of the NSW DECC is 200 µS/cm. Therefore the salinity of culture media and controls were adjusted to 200 µS/cm and this was considered as the "normal salinity" for *D. carinata*.

New cladoceran cultures were initiated each week on Wednesday using < 24-hour-old neonates. These were transferred to 2L beakers with fresh cladoceran water and food. Cultures were maintained for four weeks, at which point they were discarded and replaced by new cultures. Mass cultures of 1, 2 and 3 weeks of age were maintained throughout the study, thus ensuring a continual supply of neonates for use in the toxicity studies.

5.3.2. Experimental design

The treatments used for the multigenerational tests were 200 (the salinity of the cladoceran water) 2000, 4000, 5500 and 6300 µS/cm (based on the range finding tests of salinity acute toxicity for *D. carinata*). These test solutions were prepared by mixing appropriate amounts of filtered, UV-sterilised and aerated seawater (collected from Cronulla, NSW) with cladoceran water.

The cladocerans were exposed to the above salinity treatments over four generations starting with a parental generation (F0) and three subsequent generations (F1, F2 and F3). Each treatment consisted of 10 replicates. Each generation was terminated and a new generation commenced after at least 90% of the control animals completed their third brood. The new generations were started using third-brood neonates from the preceding generation.

On terminating each generation, all the adults and the second brood neonates that were less than 24 hours old (except for the F3 generation) were preserved in sugar formalin to prevent distortion of the carapace (Haney and Hall 1973) for later length measurements. The body-length measurements were taken from the anterior-most part of the head to the base of the tail (Koivisto and Ketola 1995), with a Leica microscope, camera and image analyser (microscope - Wild Leitz Heerbrugg, Camera - Leica DC100 and software - Leica Qwin Std v3.2.0). The mean lengths of neonates and adults were calculated for each treatment in each generation.

The survival of parental cladocerans and the number of neonates produced were recorded daily. Using the above data, the time to first brood and mean generation time T (defined as the average length of time between the birth of an individual and the birth of its offspring (Begon et al. 1996) over three generations, the mean brood size and the mean total reproduction per female (for the first three broods) in each generation were determined. The intrinsic rates of natural increase (r) of the *D. carinata* populations were calculated using the Eular-Lotka equation.

$$\Sigma\, l_x m_x e^{-rmx} = 1 \qquad 5.1$$

where x is the age in days, l_x is the number of females surviving on day x, and m_x is the number of neonates produced on day x. The 95% confidence limits of r were calculated using a Jackknife resampling procedure (Taberner et al. 1993).

The feeding experiments were conducted with 24 to 48 hour-old neonates of the F0, F1, and F2 generations, using the same salinity treatments in which they had been cultured. Each treatment consisted of four replicates, where five neonates were added to 100 mL of the test solution. An algal suspension of *Pseudokirchneriella subcapitata* was added to provide approximately 1.0 x 10^6 cells/100 mL. Only *P. subcapitata* was used (and not a mixture of *Ankistrodesmus* sp. and *P. subcapitata*) to ensure accurate enumeration of algal cells by the electronic particle counter. The beakers were incubated for 24 hours at 20°C in darkness to prevent any algal growth during the incubation period. To correct for possible growth of the algae, another four replicates of each treatment were also incubated under the same conditions but

without cladocerans. Measurements of the algal cell densities were taken at the beginning and at termination of tests. The filtration rate (F), which is the volume of media cleared of suspended particles per unit time and the ingestion rate (I), which is the number of cells consumed by an animal at a given time, for each generation at each salinity treatment were calculated using the method described by (Frost 1972).

$$F = V/n\ (\ln Ci - \ln Cf)/t - A \qquad 5.2$$

$$A = (\ln Ci - \ln Cf)/t \qquad 5.3$$

$$I = FCm \qquad 5.4$$

where F is the filtration rate (μL/ind/hr), V is the test volume (μL), Ci and Cf are the initial and final algal concentrations (cells/μL), n is the number of cladocerans per replicate and t is the feeding time (hours). A is the correction factor for changes in the blanks with final concentration Cf after time t. I is the ingestion rate (cells/ind/hr) and Cm is the mean food concentration at time t.

5.3.4. Statistical analysis

The survival of adults in all four generations was tested using Fisher's Exact test to determine whether any salinity treatments had a significant ($p \leq 0.05$) effect on parental mortality (OECD 1998).

All data were tested for homogeneity and normality using Bartlett's test and Shapiro-Wilk's tests. Log or arcsine transformation of data was performed whenever necessary, prior to further statistical analysis. The data on reproduction, brood size, time to first brood, generation time, feeding and ingestion rates were analysed using ANOVA and subjected to pair-wise analysis using either Bonferoni t tests or the Wilcoxon rank test with Bonferroni adjustment with an error rate of 0.05. Based on that, the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined.

The intrinsic rate of natural increase (r) data were analysed using Dunnett's test to compare variations between treatments in each generation (Meyer et al. 1987) and the NOEC and LOEC values were determined accordingly.

The point intercept method (Norberg-King 1998) was used to determine the salinity that caused a 25% effect (EC25). The EC25 was selected since it generally corresponds to the NOEC value (Norberg-King 1998). The statistical differences of the endpoints among the generations were determined using EC25 with the use standard error of the difference test (Sprague and Fogels 1977).

5.4. Results

5.4.1. Effects of salinity on life history traits within each generation.

The salinity treatments had no significant effect ($p > 0.05$) on parental mortality in any of the generations and therefore the data were used for further analysis without modification (OECD 1998).

The effects of elevated salinity of the various endpoints measured over time are graphically illustrated in Figures 5.1 to 5.8.

Figure 5.1 shows the variations in mean total reproduction per female for each generation. In the F0, F2 and F3 generations the 5500 and 6300 µS/cm treatments significantly ($p \leq 0.05$) reduced the total reproduction per female. However, in the F1 generation only the 6300 µS/cm treatment significantly ($p \leq 0.05$) reduced total reproduction per female.

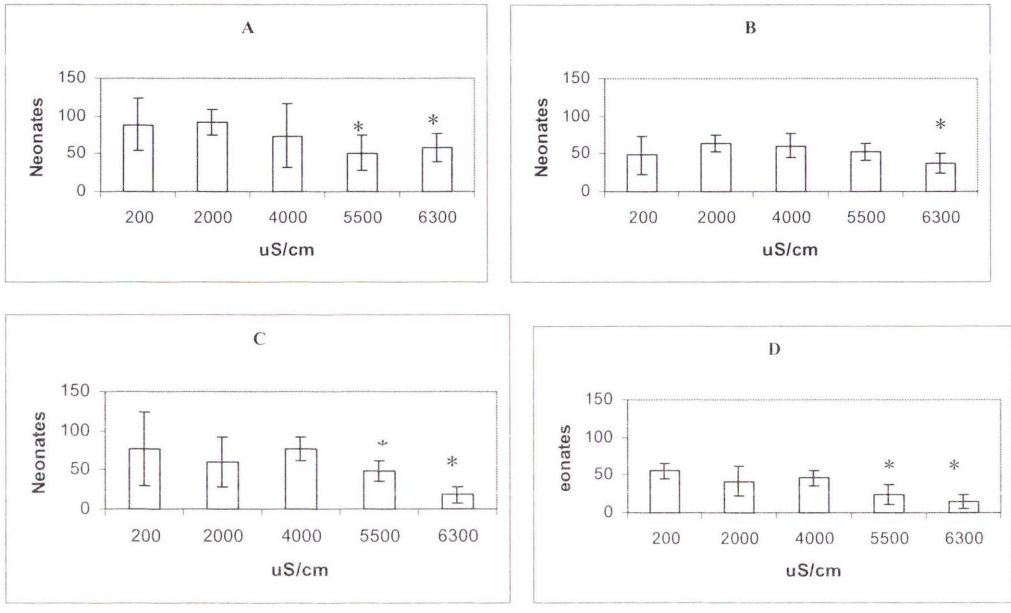

Figure 5.1: Variations in mean total reproduction per female in each generation as a function of salinity. *D. carinata* cultured in A: F0 generation, B: F1 generation, C: F2 generation, D: F3 generation. Bars are standard errors of means. * significantly different ($p < 0.05$) from the control for that generation.

The variations in mean brood sizes as a function of salinity are illustrated in Figure 5.2 for each generation. Mean brood sizes in the 5500 and 6300 µS/cm treatments in the F0 and F2 generations were significantly ($p \leq 0.05$) smaller than that of their appropriate controls (Figures 5.2A and C). No significant differences ($p > 0.05$) among treatments were observed in the F1 generation (Figure 5.2B). Mean brood size in the F3 generation in the 4000, 5500 and 6300 µS/cm treatments were significantly smaller ($p \leq 0.05$) than that of the control (Figure 5.2D).

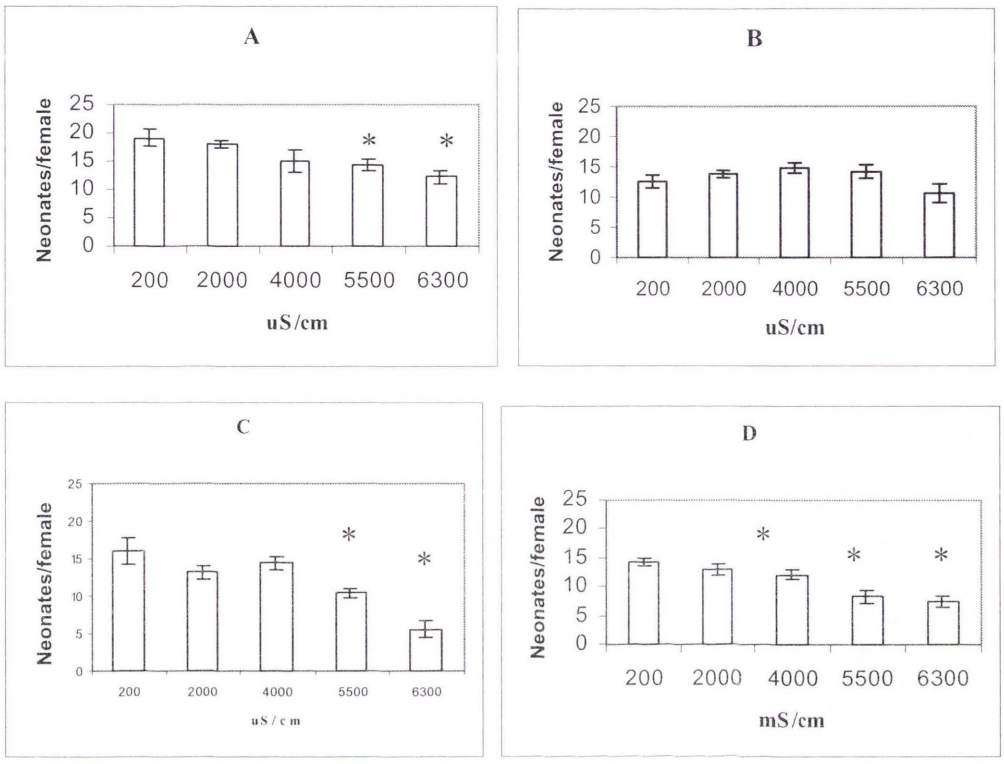

Figure 5.2: Variations in mean brood size in each generation as function of salinity, *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation, D: F3 Generation. The error bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

The variations in time to first brood and mean generation time as a function of salinity are shown in Figure 5.3 for each generation. For time to first brood, significant decreases ($p \leq 0.05$) from that of the control were observed in the 2000 µS/cm treatment in the F0 generation (Figure 5.3A). Significant ($p \leq 0.05$) increases occurred in 4000, 5500 and 6300 µS/cm treatments of the F1 generation (Figure 5.3B), in 6300 µS/cm of the F2 generation (Figure 5.3C), and in the 5500 and 6300 µS/cm treatments of the F3 generation (Figure 5.3D).

For mean generation time, none of the treatments in the F0 generation were significantly different ($p > 0.05$) from that of the control. The 5500 and 6300 µS/cm treatments of the F1 generation had significantly ($p \leq 0.05$) longer mean generation times than that of their appropriate controls (Figure 5.3B). Only the 6300 µS/cm treatment in the F2 generation (Figure 5.3C) and 5500 and 6300 µS/cm treatments in

the F3 generation (Figure 5.3D) were significantly longer ($p \leq 0.05$) than that of their appropriate controls.

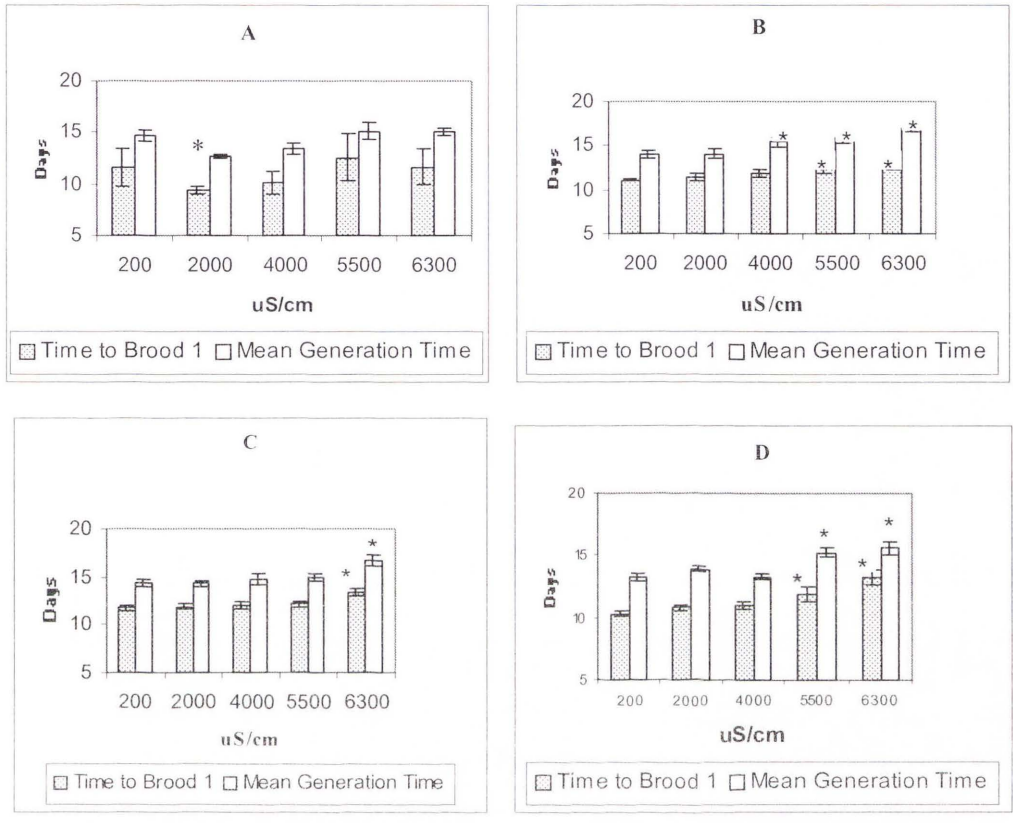

Figure 5.3: Variations in time to first brood and mean generation time as a function of salinity. The *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation, D: F3 Generation. Bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

The variations in neonatal length as a function of salinity are shown in Figure 5.4 for the F0, F1 and F2 generations. All the salinity treatments caused a significant ($p \leq 0.05$) reduction in neonatal body length compared to that of their appropriate controls (Figures 5.4A – 5.4C).

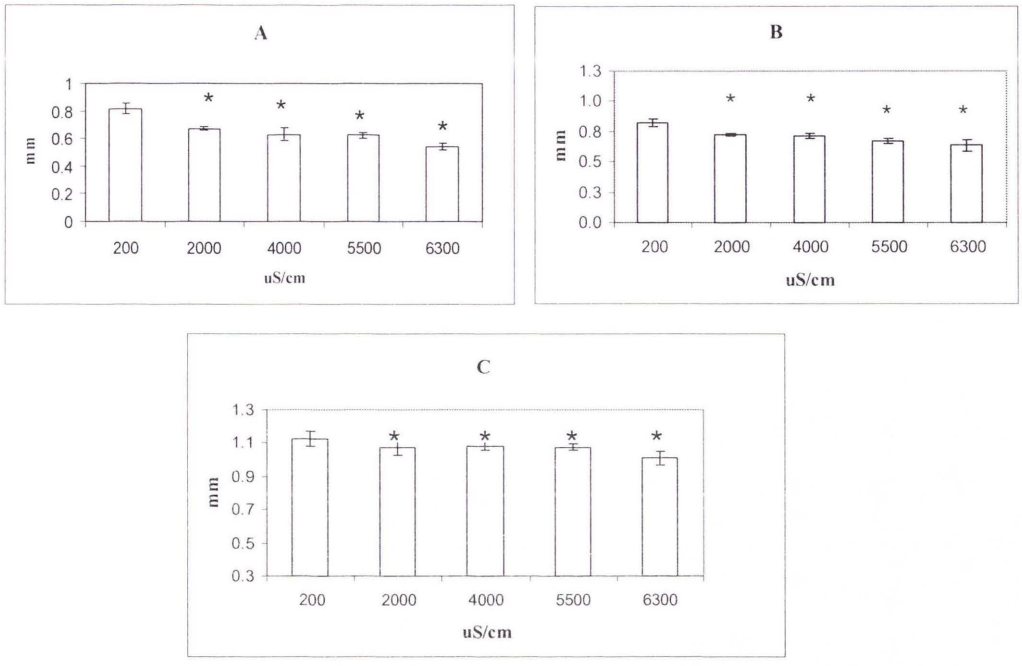

Figure 5.4: Variations in body lengths of *D. carinata* neonates of brood 2, produced in each generation as a function of salinity. *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation. The error bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

Variations in adult body lengths as a function of salinity are presented in Figure 5.5 for the F0, F1, and F2 generations. In the F0 and F2 generations, the 5500 and 6300 µS/cm treatments body lengths were significantly ($p \leq 0.05$) smaller than that of the appropriate controls. In the F1 generation, the 4000, 5500 and 6300 µS/cm treatments, body lengths were significantly smaller ($p \leq 0.05$) than that of the appropriate controls (Figure 5.5B).

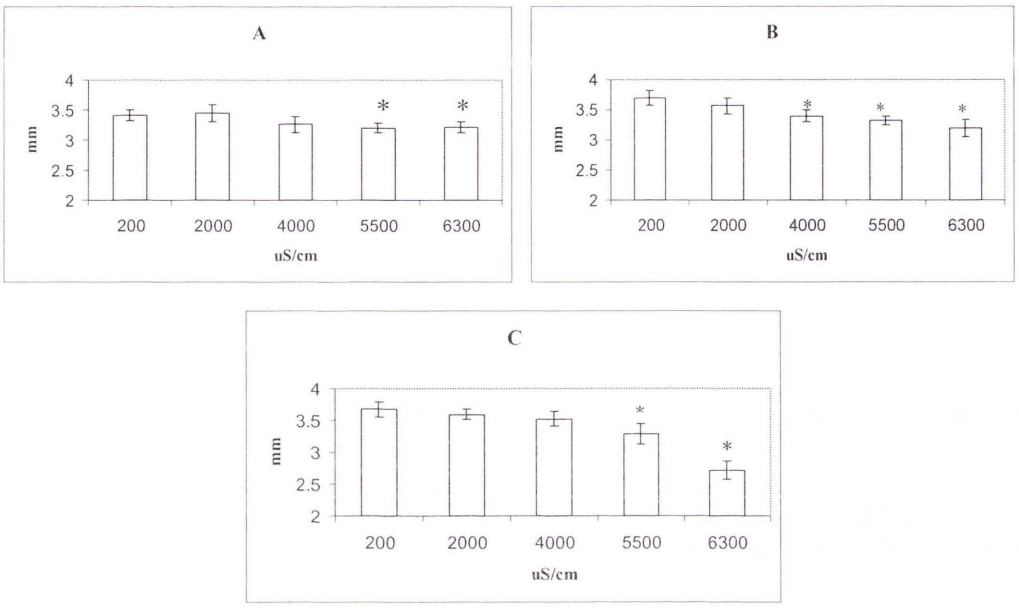

Figure 5.5: Variations in body lengths of *D. carinata* adults in each generation as a function of the salinity. *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation. Bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

Variations in *D. carinata* filtration rates as a function of salinity are shown in Figure 5.6 for the F0 – F2 generations. No significant differences ($p > 0.05$) were observed in the filtration rates of *D. carinata* in the F0 generation (Figure 5.6A). For the F1 generation, in the 5500 and 6300 µS/cm treatments, filtration rates were significantly reduced ($p \leq 0.05$) compared to that of the control (Figure 5.6B). For the F2 generation in the 4000, 5500 and 6300 µS/cm treatments, filtration rates were significantly ($p \leq 0.05$) lower than that of the control (Figure 5.6C).

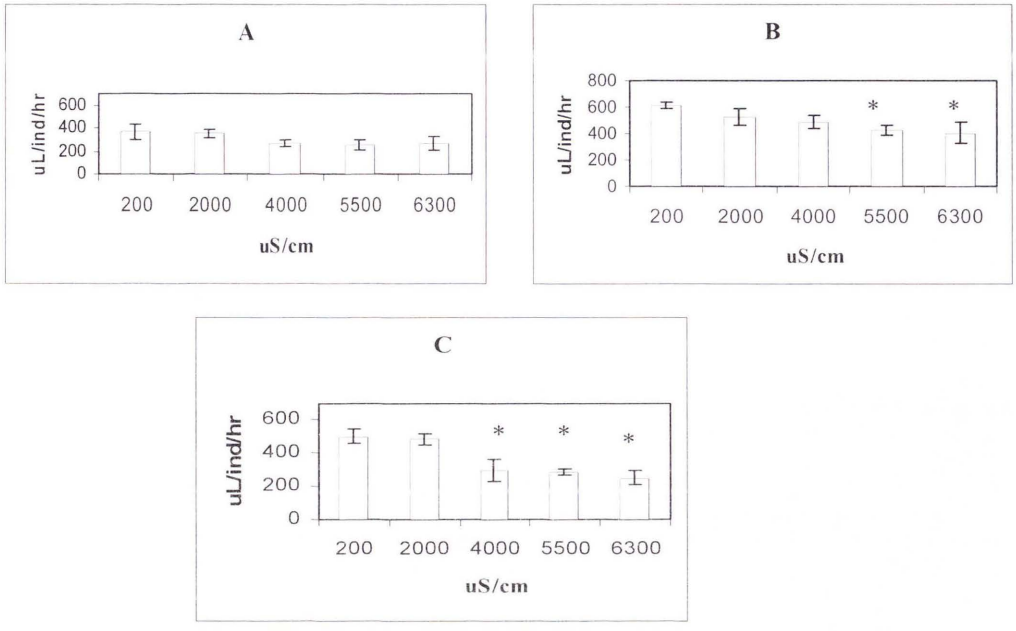

Figure 5.6: Variations in filtration rates of *D. carinata* in each generation as a function of salinity. *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation. Bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

Variations in *D. carinata* algal ingestion rates as a function of salinity are shown in Figure 5.7. In the F0 generation, ingestion rates in the 6300 µS/cm treatment were significantly ($p \leq 0.05$) reduced compared to that of the control (Figure 5.7A) while there were no significant ($p > 0.05$) differences in the F1 generation (Figure 5.7B). In the F2 generation, ingestion rates in the 4000, 5500 and 6300 µS/cm treatments were significantly ($p \leq 0.05$) reduced compared to that of the control (Figure 5.7C).

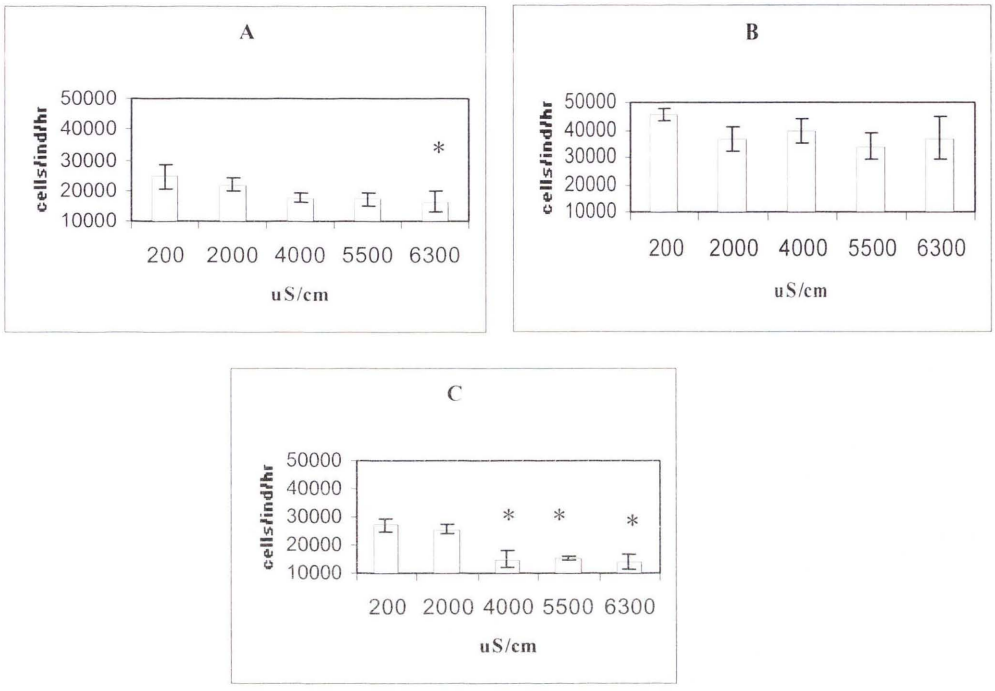

Figure 5.7 Variations in ingestion rate of *D. carinata* in each generation as a function of salinity. *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation. Bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

Variations in intrinsic rate of natural increase (r) as a function of salinity are presented in Figure 5.8. The values of the F0 generation did not significantly ($p > 0.05$) differ from that of the control (Figure 5.8A). The r value in the 5500 and 6300 µS/cm treatments were significantly ($p \leq 0.05$) lower than those of the appropriate controls in the F1, F2 and F3 generations (Figures 5.8B – 5.8D).

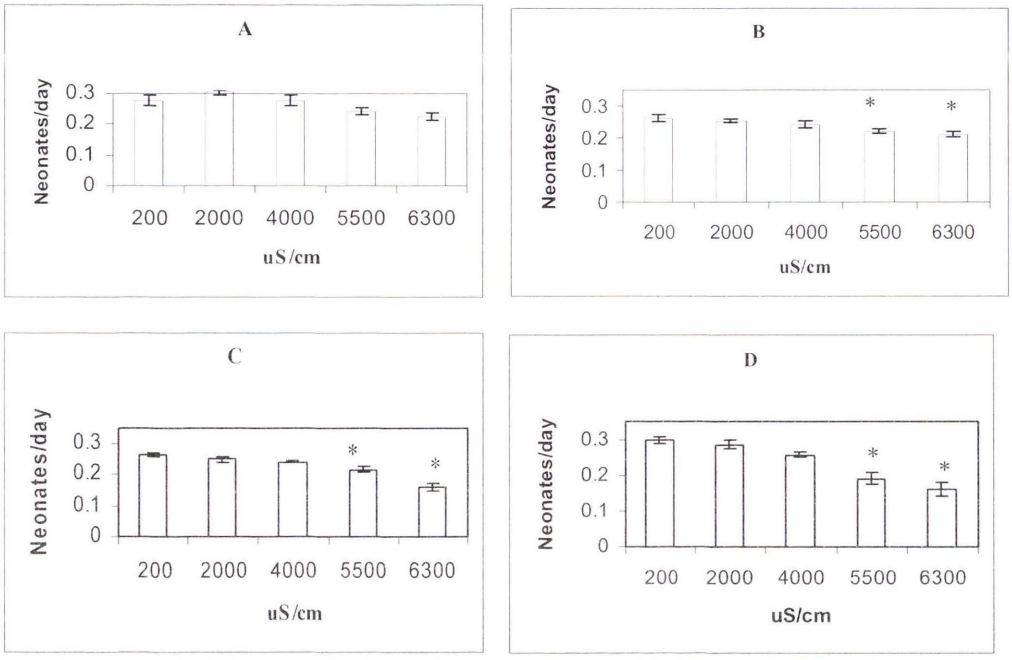

Figure 5.8: Variations in intrinsic rate of natural increase (r) of *D. carinata* in each generation as a function of salinity. *D. carinata* were cultured in A: F0 Generation, B: F1 Generation, C: F2 Generation, D: F3 Generation. Bars are standard errors of means. * Significantly different ($p \leq 0.05$) from the control for that generation.

5.4.2. Comparison of the effect of salinity across generations

To determine if the effect of salinity on the life history traits of *D. carinata* changed over time, two different approaches were used. Firstly, the NOEC and LOEC values for each generation were compared (Table 5.3). However, this approach does not permit statistical comparisons between the effects that salinity had on each generation. Therefore, a second approach in which the salinities that caused a 25% deleterious effect (EC25) were calculated for each life history trait at each generation (Table 5.4) and these were compared using the standard error of the difference test (Sprague and Fogels 1977). The intrinsic rate of natural increase values of each generation (F0 – F3) were also compared (Sprague and Fogels 1977) to determine the variations among the generations and are presented in Table 5.5.

Table 5.3: The no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) values of salinity to selected life history traits of *D. carinata* in each generation.

Endpoint		F0	F1	F2	F3
Total Reproduction	NOEC	4000	>6300[1]	5500	4000
	LOEC	5500	>6300	6300	5500
Mean brood size	NOEC	2000	>6300	4000	2000
	LOEC	4000	>6300	5500	4000
Time to first brood	NOEC	BLT[2]	4000	5500	4000
	LOEC	2000	5500	6300	5500
Neonate length	NOEC	BLT	BLT	BLT	ND[3]
	LOEC	2000	2000	2000	ND
Adult length	NOEC	4000	2000	4000	ND
	LOEC	5500	4000	5500	ND
Filtration rate	NOEC	>6300	4000	2000	ND
	LOEC	>6300	5500	4000	ND
Ingestion rate	NOEC	5500	>6300	2000	ND
	LOEC	6300	>6300	4000	ND
Mean generation time	NOEC	>6300	2000	5500	4000
	LOEC	>6300	4000	6300	5500
Intrinsic rate of nat. increase	NOEC	>6300	4000	4000	4000
	LOEC	>6300	5500	5500	5500

[1]. > 6300 = greater than the highest tested salinity treatment. [2]. BLT = below the lowest tested salinity treatment. [3]. ND = Not determined.

In terms of NOEC and LOEC values, only for time to first brood and filtration rate were there any consistent trends in the effect that salinity had over time (Table 5.3). For time to first brood the NOEC and LOEC values increased between F0 and F1 and then remained similar; while for filtration rate NOEC and LOEC values decreased over time.

The only life history trait where there were significant ($p \leq 0.05$) changes in the EC25 values between generations was mean total reproduction per female (Table 5.4). The mean total reproduction per female of the F0 generation was significantly smaller ($p \leq 0.05$) than that of the F1 and F2 generations, but significantly larger ($p \leq 0.05$) than that for the F3 generation (Table 5.4). Thus there was no consistent trend over time. The mean intrinsic rate of natural increase values were significantly smaller ($p \leq 0.05$) in the F1 and F2 generations compared to that of the F0 (Table 5.5). However, the F3 generation was not significantly different ($p > 0.05$) from the F0 generation.

Table 5.4: Salinities which caused a 25% deleterious effect on selected life history traits of *Daphnia carinata* for generations F0 to F3. (confidence intervals are indicated in parantheses)

Life history trait	Measure of toxicity	F0	F1	F2	F3
Total reproduction per female	EC25	4474 (2502–5429)	5958* (5351-6249)	4761* (4052–5695)	4186* (3260-4696)
Mean brood size	EC25	5297 (2882-6105)	>6300[a]	4825 (3524-5609)	4705 (2499-6128)
Neonate length	EC25	5428 (3740-5775)	>6300	>6300	ND[b]
Adult length	EC25	>6300	>6300	6242 (6081-6295)	ND
Filtration rate	EC25	3907 (2212-6001)	4680 (2781-5820)	3179 (2459-4348)	ND
Ingestion rate	EC25	3656 (2155-5958)	>6300	3001 (2292-3645)	ND

[a] – values with a greater than sign are where the EC25 values was greater than the highest tested salinity. Such values cannot be compared statistically to those in other generations. [b] ND = Not determined. * indicates values that are significantly ($p \leq 0.05$) different to the F0 value for that life history trait.

Table 5.5: The variations of mean intrinsic rate of natural increase of *D. carinata* in generations of F0 – F3.

Life history trait	F0	F1	F2	F3
Intrinsic rate of natural increase	0.27 (0.25 – 0.28)	0.24* (0.23 – 0.25)	0.23* (0.21 – 0.24)	0.24 (0.18 – 0.30)

* indicates values that are significantly ($P \leq 0.05$) different to the F0 value

5.5. Discussion

The two factors that control the intrinsic rate of natural increase are adult survival and reproduction (equation 5.1). The survival of adults was not affected at any of the salinities tested irrespective of the generation. However, the time to first brood and mean generation time were both detrimentally affected by the higher salinity treatments, with the exception of the F0 generation. Significant delays in reproduction mean that the intrinsic rate of natural population increase should be reduced and this was observed for salinities $\geq$ 5500 µS/cm (Figure 5.8). The intrinsic rate of natural increase denotes the favourability of the environment for growth and reproduction, and fitness of the species in the environment. In the present study it is clear that

salinities at or above 5500 µS/cm do not provide a favourable environment for survival.

Reduced feeding capability by the cladocerans due to the reduction in filtration and ingestion rates could have negative impacts on the scope for growth and fitness of the organisms (Baillieul et al. 1996). It has also been shown that undernourished cladocerans are more sensitive to toxicants(Rose et al. 2002a). Reduced feeding could be due to either direct or indirect effects of salinity. Direct effects include the fact that increased salinity suppresses appetite or reduces the quality of the algal food. Indirect effects include the fact that salinity reduced the length of neonates (Figure 5.4) and thus their overall size and smaller cladocerans would tend to eat less food than larger cladocerans.

Freshwater cladocerans have excellent osmoregulatory abilities to maintain their haemolymph at hyperosmotic (i.e. more saline) concentrations compared to that of the external environment (Aladin and Potts 1995). Cladocerans in the control freshwater medium (200 µS/cm) expend energy on this active osmoregulatory process, which could otherwise be used for other vital processes such as reproduction and growth. The present study reveals that in the lower salinity treatments (i.e., 2000 and 4000 µS/cm) undesirable impacts are not clearly visible in terms of the life history traits studied. On the contrary, slightly, but not significantly ($p > 0.05$) higher reproduction (in terms of mean total reproduction per female and mean brood size) was sometimes observed in moderately elevated salinity treatments. This occurred in the 2000 µS/cm treatment in the F0 generation, in the 2000 and 4000 µS/cm treatments of the F1 generation and in the 4000 µS/cm treatment of the F2 generation for mean total reproduction per female. Non-significant ($p > 0.05$) increases also occurred in the 2000, 4000 and 5500 µS/cm treatments in the F1 generation for mean brood size. These slight increases are consistent with the cladocerans having to expend less energy on osmoregulation (as the ambient salinity approaches their internal salinity) and having more energy available for reproduction. Arner and Koivisto (1993), recorded population growth rate and time to each brood of *D. magna* were optimum at a salinity of 6500 µS/cm which is higher than the results of the present study.

However, this variation could be as a result of the differences in two species used in the two studies.

In higher salinity external environments, internal body fluid and haemolymph become iso-osmotic, and if the salinity increases further, it can cause detrimental effects, including lethality. Based on the results of the present study, salinities higher than 5500 μS/cm in general caused significant deleterious impacts on *D. carinata*, and the cladocerans appear to be increasingly stressed. Total reproduction, time to first brood, intrinsic rate of natural increase, filtration and ingestion rates were compromised with increasing salinity as it becomes increasingly difficult for the cladoceran to cope physiologically. Behavioural changes may also play a role, as indicated in the lower feeding and ingestion rates at high salinity, which could be a precautionary measure to reduce salt uptake.

The salinity treatments in the present study used sea water, because Australian inland saline waters have a similar ionic composition to that of sea water (Bayly and Williams 1972). Sea water is mainly dominated by less toxic monovalant ions such as Na^+ and Cl^- ions and is relatively low in the more toxic divalent ions. Because of this, unlike some other inland saline waters such as mine drains, salinity seems to act as a physiological stressor rather than a conventional toxicant. This explains the lack of salinity-induced adaptations through the generations other than mean total reproduction in the F1, F2 and F3 generations, and the variations in intrinsic rate of natural increase among the F1 and F2 generations. Tanaka and Nakanishi (2002), working on the *p*-nonylphenol toxicity on *D. galeata,* observed a similar trend but attempted to describe the trans-generational effects based on high still-births and neonatal mortality. Genetic characteristics could also play a role in differences in responses to toxicants (Teschner 1995) but the present study was conducted with cultures that reproduced parthenogenetically over an extended period and therefore any genetic variation within these *D. carinata* in the present study would be minimal.

The parameters that provide better indications of the impacts of salinity within each generation, in descending order, are neonatal length, adult length, intrinsic rate of natural increase, filtration rate and ingestion rate. The effects between generations

were better displayed by the mean total reproduction and mean intrinsic rate of natural increase.

5.6. Conclusions

There were no significant effects of salinity to *D. carinata* up to 4000 μS/cm over the generations studied except for neonatal lengths. Generally, at salinities ≥ 5500 μS/cm, reproductive ability, adult body lengths, intrinsic rate of natural increase, filtration rates and ingestion rates of *D. carinata* were significantly reduced. However, for some endpoints, such as neonatal length and time to first brood, significant effects occurred at 4000 μS/cm and, for length, at 2000 μS/cm. Slightly greater adult length, brood size and intrinsic rate of natural increase at 2000 μS/cm in the F0 generation indicates that the energy saved from osmoregulation can be used for the other functions such as growth and reproduction. Significant delays in the production of neonates also occurred with increasing salinities.The sensitivity of *D. carinata* in terms of total reproduction per female and the mean intrinsic rate of natural increase both increased due to exposure to elevated salinity. *D. carinata* did not develop tolerance to increased levels of salinity over the generations for any of the endpoints measured. The results of the present study showed that, salinisation of inland waters could have long-term detrimental impacts on cladoceran populations and hence may affect zooplanktivorous species in the aquatic food chain.

Chapter 6

Acute toxicity of salinity and the pesticides atrazine, chlorpyrifos and molinate individually and as mixtures to the freshwater cladoceran *Daphnia carinata*

6.1. Abstract

Salinisation of inland water bodies occurs in many parts of the world. This issue is serious in Australia, especially in agricultural areas which are also subject to pesticide pollution. Aquatic organisms are, therefore, likely to be exposed to mixtures of pesticides and salinity. However, there is little information available on the toxicities of combinations of pesticides and salinity on freshwater organisms. The present study determined the concentrations of salinity, atrazine, chlorpyrifos and molinate that each caused immobilisation of 50% (EC50 immobilisation) of the population of the freshwater cladoceran, *Daphnia carinata*. The toxicity of mixtures of each pesticide with salinity, equitoxic mixtures of the pesticides, and finally mixtures of pesticides and salinity were determined and expressed as toxic units (TU). The mean toxicities of salinity, atrazine, molinate and chlorpyrifos acting individually were 8800 μS/cm, 42 mg/L, 25 mg/L and 0.21 μg/L, respectively. The mixtures of atrazine/salinity generally caused toxicity consistent with concentration addition (additivity). The toxicities of the chlorpyrifos/salinity and molinate/salinity mixtures were generally consistent with antagonism. For the equitoxic mixtures of pesticides (i.e., atrazine/molinate, atrazine/chlorpyrifos and atrazine/molinate/chlorpyrifos), toxicity was consistent with antagonism below 1 TU and was then consistent with additivity and synergism at higher concentrations. Mixtures of multiple pesticides and salinity were predominantly additive. There were fourteen mixtures tested giving sixty-nine treatment combinations of which 50% of the combinations were consistent with additivity while 10% and 40% of the combinations were consistent with synergism and antagonism, respectively. These results suggest the need for incorporating mixture toxicities into the derivation of water quality guidelines.

6.2. Introduction

The issue of increasing inland salinity is a world wide-problem (Williams 1987). Australia in particular suffers from salinisation of inland water bodies and the problem predominantly occurs in agricultural areas (Pinder et al. 2005) where the extensive use of pesticides is also prevalent (Muschall and Warne 2003). Therefore, it is important to understand the combined effects of salinity and pesticides on the aquatic biota. Information on the toxic effects of salinity on individual pesticides is fairly well documented (Donham et al. 2006; Hall and Anderson 1995; Hall et al. 1995). However, most of the studies were confined to estuarine or euryhaline organisms whose habitats are saline (Hall and Anderson 1995) and not on freshwater biota that are likely to be affected by increased salinity. The little information available on freshwater species is restricted to mixture toxicity effects of single pesticides with salinity (Hansen 1972) and not on mixtures of pesticides and salinity, which represent a more realistic situation in the natural environment.

The importance of studying the toxic effects of mixtures of chemicals on organisms has been widely discussed by the scientific community. In particular, emphasis has been placed on the need to incorporate mixture toxicity information in drafting and formulating environmental quality standards (Calamari and Vighi 1992; Chevre et al. 2006; ECETOC 2001; Junghans 2004; Junghans et al. 2006; Warne 2003). Yet despite acceptance of the need for toxicity data on mixtures, the vast majority of toxicity testing still examines the effects of individual chemicals.

It is important to study toxicities of mixtures of salinity and pesticides using a representative test organism in order to understand their effects. Cladocerans are among the most widely used test animals for ecotoxicological studies and they represent the primary consumer trophic level in freshwater food chains. The freshwater cladoceran, *Daphnia carinata*, which is found in Australia, is considered a suitable species as it is distributed in salinity affected areas (Benzie 1988). *D. carinata* has been used in toxicological studies related to salinity alone (Hall and

Burns 2002; Kefford et al. 2002) and pesticides (Barry et al. 1995; Zalizniak and Nugegoda 2006).

Given the potential for Australian freshwater organisms to be exposed to mixtures of pesticides in combination with salinity, and the lack of toxicity data, the objectives of the present study were to 1) determine the acute toxicity of atrazine, chlorpyrifos, molinate and salinity individually to *D. carinata*, and 2) determine the acute toxicity of various mixtures of the above chemicals and salinity to this species.

6.3. Materials and Methods

6.3.1. Test species

The freshwater cladoceran, *Daphnia carinata*, was selected as it has been widely used for toxicity testing (Chandini 1989; Phyu, Warne et al. 2004; Zalizniak and Nugegoda 2006), is easy to maintain and culture; has a relatively short life cycle (i.e., it breeds after approximately 10 days and lives for approximately 60-80 days); represents the trophic level of primary consumers and as such is linked to higher levels of food web via zooplanktivorous fish. The *D. carinata* populations used in the present study were obtained from the Centre for Ecotoxicology, Lidcombe, NSW, Australia, which were originally sourced from the NSW Fisheries Research Station at Narrandera, NSW, Australia (Moreno Julli, NSW DECC, *pers. comm.*).

6.3.2. Toxicants

The pesticides used in the study were atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1, 3, 5-triazine-2, 4-diamine; CAS No-1912-24-9), chlorpyrifos (O,O-Diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CAS No. 2921-88-2) and molinate (s-ethyl *N*, *N*-hexa methylene thiocarbamate; CAS No-2212-67-1). All three chemicals were reagent-grade technical chemicals (≥97% purity). These three pesticides were selected since they are among the commonly used pesticides in Australian agriculture (Bowmer et al. 1998).

Sea water collected from Cronulla (NSW, Australia) was used as the source of salinity in the toxicity tests for the effect of salinity. Sea water was used as the ionic composition of Australian inland salt is essentially identical to that of sea water (Bayly and Williams 1972).

Stock and working solutions of pesticides were prepared in analytical grade (99% purity) acetone as the carrier solvent. The stock solutions were stored in a freezer at -4°C and all working stock solutions were made immediately prior to use

6.3.3. *D. carinata* cultures

The culturing of *D. carinata* was carried out according to the methods specified in the Methods Manual of the Ecotoxicology Section of the NSW DECC. Detailed culture techniques are described in Chapter 5 of this thesis.

6.3.4. Experimental design

Toxicity experiments were conducted in four groups. An overall description of the experimental design of these four groups is provided below. This is followed by details of the experimental design.

6.3.4.1. Group 1 experiments

Group 1 experiments consisted of acute toxicity tests for salinity, atrazine, molinate and chlorpyrifos – each tested separately. Each experiment consisted of a control and five treatments of increasing concentrations of a pesticide or salinity. Based on the results of these tests, the concentrations of individual chemicals and salinity to be used in the mixture tests were determined.

6.3.4.2. Group 2 experiments

Group 2 experiments determined the toxicity of mixtures of salinity with individual pesticides (i.e., salinity and atrazine, salinity and chlorpyrifos, and salinity and molinate). The treatments in these experiments used a fixed concentration of a pesticide with increasing concentrations of salinity (Table 6.1). The experiments were designed so that the following pesticide concentrations were tested:

- 0.5 TU for atrazine
- 1 TU for atrazine
- 0.5 TU for molinate
- 1 TU for molinate
- 1 TU for chlorpyrifos
- 1.5 TU for chlorpyrifos.

The contribution of toxic units for each pesticide was determined based on the preliminary range finding experiments. The mixture of chlorpyrifos and salinity was shown to be conform to antagonism and therefore 1 and 1.5 TU for chlorpyrifos were considered as appropriate. This will not lead to any overestimation of the type of toxic joint action, as the TU values of the components of the mixture are accounted for in determining the predicted toxicity and therefore whether the mixture was additive, synergistic or antagonistic. The actual concentrations (in terms of TUs) used in Group 2 experiments are presented in Table 6.1.

Table 6.1: Concentrations of each treatment of each pesticide and salinity mixture expressed in toxic units (TUs) and the contributions of salinity to the treatment. The pesticide in each mixture had a fixed concentration, while that of salinity increased.

Treatment	Toxic contribution from salinity	Concentration of mixtures (TUs)		
		pesticide present at 0.5 TU	pesticide present at 1 TU	pesticide present at 1.5 TU
Control	0	0	0	0
1	0	0.5	1	1.5
2	0.46	0.96	1.46	1.96
3	0.68	1.18	1.68	2.18
4	0.79	1.29	1.79	2.29
5	0.91	1.41	1.91	2.41
6	1.14	1.64	2.14	2.64

6.3.4.3. Group 3 experiments

Group 3 experiments consisted of mixtures of all possible combinations of the pesticides (i.e., atrazine/molinate, atrazine/chlorpyrifos, chlorpyrifos/molinate, and atrazine/molinate/chlorpyrifos). Each experiment consisted of a control and six pesticide mixture treatments of increasing TUs (Table 6.2). The mixtures were equitoxic – i.e., each pesticide was present in the mixture at the same proportion of its own EC50 value measured individually. Thus, in Treatment 1 of the atrazine/chlorpyrifos/molinate test where the total TU is 0.24, each pesticide would be present at a concentration of 0.08 TU of its EC50 value.

Table 6.2: Concentrations of each treatment of the equitoxic pesticide mixtures expressed as toxic units (TUs).

Treatment	Concentration of mixtures (TUs)			
	atrazine/ molinate mixture	**atrazine/ chlorpyrifos**	**chlorpyrifos/ molinate**	**atrazine/ chlorpyrifos/ molinate**
Control	0	0	0	0
1	0.24	0.25	0.24	0.24
2	0.48	0.49	0.49	0.49
3	0.95	0.98	0.98	0.97
4	1.19	1.23	1.22	1.21
5	1.43	1.47	1.47	1.46
6	1.91	1.96	1.96	1.94

6.3.4.4. Group 4 experiments

Group 4 experiments consisted of combinations of two or more pesticides with salinity (i.e., atrazine / molinate / salinity, atrazine / chlorpyrifos/ salinity, molinate / chlorpyrifos/salinity, and atrazine/molinate/chlorpyrifos/salinity). Each experiment consisted of a control and six concentrations of the mixture. All treatments of each mixture contained a fixed concentration of pesticide (i.e., a total of 1 TU) but increasing salinity (Table 6.3). The pesticide component of each mixture was present as an equitoxic mixture – thus in the binary mixtures each pesticide was present at 0.5 TU and in the tertiary mixtures each pesticide was present at 0.33 TU.

Table 6.3: The concentration of each treatment of each pesticide and salinity mixture expressed as toxic units (TUs) and the contribution to the treatment of salinity. Pesticide mixtures had fixed concentrations, while that of salinity increased.

Treatment	Toxic contribution from salinity	Concentration of mixtures (TUs)			
		atrazine/ molinate/ salinity	atrazine/ chlorpyrifos/ salinity	chlorpyrifos / molinate/ salinity	atrazine/ molinate/ chlorpyrifos /salinity
Control	0	0	0	0	0
1	0	0.95	0.98	0.98	0.97
2	0.46	1.18	1.21	1.21	1.20
3	0.68	1.41	1.44	1.44	1.43
4	0.79	1.64	1.67	1.67	1.67
5	0.91	1.87	1.89	1.89	1.89
6	1.14	2.09	2.12	2.12	2.11

6.3.5. Test method

The acute toxicity testing method used was based on USEPA protocols (USEPA 2002) except that an Australian cladoceran species, *D. carinata* was used; animals were maintained in mass cultures, and only algae were provided as food.

The acute toxicity tests were designed to subject the < 24-hour-old neonates to geometrically increasing concentrations of toxicants and their mixtures over a 48-hour period. Immobilisation was the test endpoint. Immobilisation is defined as the absence of visible movement of the antennae and appendages, except for minor spontaneous, random activity, within 15 seconds of gentle agitation of the test solution (ASTM 1998).

Each acute toxicity test consisted of five concentrations for individual toxicants and six concentrations for toxicant mixtures, together with a control and a solvent control where appropriate. For the salinity-only tests, each treatment was conducted in pentuplicate, while for tests using the pesticides and mixtures of salinity and pesticides, each treatment was conducted eight times. For all tests four replicates of each treatment had neonates randomly allocated, and the fifth replicate was used solely to measure the physicochemical properties of the test solution (i.e., pH, temperature, dissolved oxygen and salinity) and therefore no neonates were added. For the pesticide and mixture tests, the sixth to eighth replicates were used for

chemical analysis of the toxicant(s), which were measured at the beginning and at the end of each test. Physico-chemical parameters (i.e., pH, temperature, conductivity and dissolved oxygen) of the test solutions were measured and recorded prior to test commencement and at 48 hours. The test solutions were not aerated and the cladocerans were not fed during the tests. Neonate immobilisation was counted at 48 hours.

All toxicity tests were conducted in 214-mL screw-capped clear glass bottles. This was to minimise loss of the chemicals through volatilisation. Test solutions were not renewed during the acute toxicity tests.

A reference toxicity test, using sea water, was conducted with each batch of neonates used in the present study. Results of the reference toxicity tests were compared to a cumulative summary (Cusum) chart to assess the acceptability of each batch of test organisms. Cusum charts were established following the methods established by the Environment Canada (1990).

Toxicity tests were considered valid if: control mortality did not exceed 10% at the end of the test; the dissolved oxygen concentration of the test solutions did not fall below 3 mg/L; the reference toxicant test results for that batch of neonates were within two standard deviations of the long-term average value obtained from the Cusum chart (Environment Canada 1990); the percentage trim used to calculate the EC50 value was less than 30%; and the test temperature of 20°C did not deviate by more than 3°C during the test.

Three valid acute toxicity tests were conducted for each of the pesticides and an average EC50 value was determined for each pesticide. Since the salinity toxicity tests were conducted throughout the study, an average EC50 value was obtained from 19 toxicity tests. Mean EC50 values were determined for each pesticide and salinity to obtain an accurate estimate of the EC50 value of each toxicant that would be used in the subsequent mixture toxicity tests.

Acute toxicity tests for mixtures were conducted using the same methods that were used for testing the toxicity of individual chemicals, except that the test solutions were different.

6.3.6. Evaluation of mixture toxicity

The toxicity of individual components in mixtures and the mixtures themselves were expressed as toxic units (TUs) using the method described by Brown (1968). Typically the TU is calculated using,

$$TU_i = \frac{C_i}{EC_{p_i}} \qquad (1)$$

where the subscript denotes the component 'i' of a mixture, while C_i is the aqueous concentration of component 'i' in a mixture and ECp_i is the aqueous concentration of the component acting individually, which will cause a given toxic effect (e.g., LC50, EC20). In the present study the term ECp_i was always the EC50 (immobilisation) value for *D. carinata*. Thus TU values of 0.5 and 1 for individual chemicals mean that they are present in a mixture at 0.5 times their EC50 value and at their EC50 values, respectively.

The Plackett and Hewlett (1952) mixture classification scheme has four types of toxic interactions (Table 6.4). These are simple similar (more usually referred to as concentration addition, CA), independent (also more usually referred to as response addition, RA), complex similar and dependent joint action. Mathematical equations have been developed to express only CA and RA.

Table 6.4: The four types of joint actions for mixtures developed by Plackett and Hewlett (1952).

	Similar Joint Action	**Dissimilar Joint Action**
Non-interactive	Simple similar (concentration addition, CA)	Independent (response addition, RA)
Interactive	Complex similar	Dependent

Theoretically the CA type of joint interaction should only occur when a mixture consists of chemicals with the same mechanism of action and the RA is the type of additive joint interaction that should apply when mixtures contain chemicals with different mechanisms of action. As the three pesticides and salinity all have different mechanisms of action, theoretically, they should be modelled using the RA approach. However, we used the CA approach, as a number of laboratory and field-based studies have shown that CA overestimated the effects and yielded slightly higher estimates of the toxicity of mixtures than RA when the chemicals had different mechanisms of action (Faust, Altenberger et al. 1994; Warne and Hawker 1995; Ross and Warne 1997; Backhaus, Altenburger et al. 2000; Backhaus, Altenburger et al. 2000; Deneer 2000; Dyer, White-Hull et al. 2000; Chevre, Loepfe et al. 2006; Junghans, Backhaus et al. 2006). It is important to use the precautionary approach but not to be over protective in developing water quality guidelines, hence the CA is the preferred model over the RA model in the present study as the latter model would underestimate the combined effects of mixtures (Junghans et al. 2006).

The results of the mixture toxicity experiments were plotted as illustrated in Figure 6.1. In this figure the concentration of the mixture that causes a certain percentage of immobilisation is expressed as toxic units. The solid line is the additivity line, which links the points 0 TU, 0% immobilisation; 0.5 TU, 25% immobilisation; 1 TU, 50% immobilisation; and 2 TU, 100% immobilisation. As indicated in ECETOC (2001), less than 30% deviation from expected additivity is considered as conforming to additivity and more than 30% deviation conforming either to antagonism or synergism. The interpretation of mixture toxicity in the present study will be based on the above model. The two dashed lines (Figure 6.1) indicate 30% deviation from additivity and mixtures that lie to the right of the lower dashed line are classified as antagonistic, while those that lie to the left of the upper dashed line are classified as synergistic. Mixtures that conform to CA will lie in between the two dashed lines.

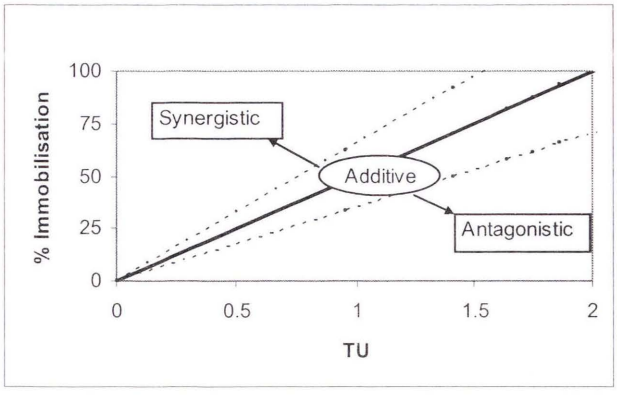

Figure 6.1: An example of the plots used to indicate the type of toxic interaction that occurs within the mixtures. The concentrations of the mixture (expressed in toxic units, TUs) that cause certain % immobilisation are plotted. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

6.3.7. Chemical analysis

Chemical analysis of test solutions was conducted at the beginning and on completion of the test to determine the loss of pesticides during the test. The concentrations of the pesticides were determined using the NSWEPA screening and extraction method for volatile and semi-volatile organic compounds. Extraction consisted of mixing a 50-mL aliquot from each treatment with 50 mL of dichloromethane in a 200-mL separatory funnel and shaking for two minutes with periodic venting. Two sequential extractions were conducted for each sample, and the extracts combined. Each combined extract was dewatered by passing it through a 5g bed of high-purity anhydrous Na_2SO_4. The dewatered extracts were concentrated down to 1 mL by evaporation, using nitrogen gas under low temperature (30°C). Solvent exchange was conducted with 4 mL acetone prior to GC analysis.

Chemical analyses were conducted on a gas chromatograph (HP 5890 series II Plus, with HP 3365 Series II Chemstation software) with a nitrogen phosphorus detector. The capillary column used for the analysis had a length of 30 m, an internal diameter of 0.25 mm, and was coated with 0.25-μm thick DB5 stationary phase. Injector port

and detector temperatures were 250 and 220°C, respectively. Nitrogen was used as the carrier gas. The temperature programme was set to have an initial column temperature of 100°C for 1 min, which was raised to 275°C at the rate of 4°C per minute and held for 1 minute. A 1 μL sample injection volume was used throughout the analyses. Calibration standards were prepared for each chemical from the stock solutions and injected after every 10 injections of the samples. The respective standard curves for each chemical were prepared and sample concentrations were determined from these.

6.3.8. Calculations and statistical analysis

The concentration of each toxicant that immobilised 50% of the test organisms (EC50 immobilisation value) and its 95% confidence limits were determined using the Trimmed Spearman-Karber method (Hamilton et al. 1977).

6.4. Results

The range of measured values of the physico-chemical parameters i.e pH (6.5 – 8.5), and electrical conductivity (190 – 200 μS/cm), temperature (20 – 21°C) and dissolved oxygen (4.0 – 7.5 mg/L) were within the acceptable limits.

6.4.1. Reference toxicant tests

The salinity reference toxicity data are presented in Figure 6.2. The EC50 values were within two standard deviations of the long-term mean EC50 value. The variations in sensitivity of the *D. carinata* to salinity over the duration of the toxicity tests were acceptable and thus the data generated for all tests were valid. These results also indicate that the sea water (used for salinity treatments) quality is uniform and its toxicity did not vary between tests.

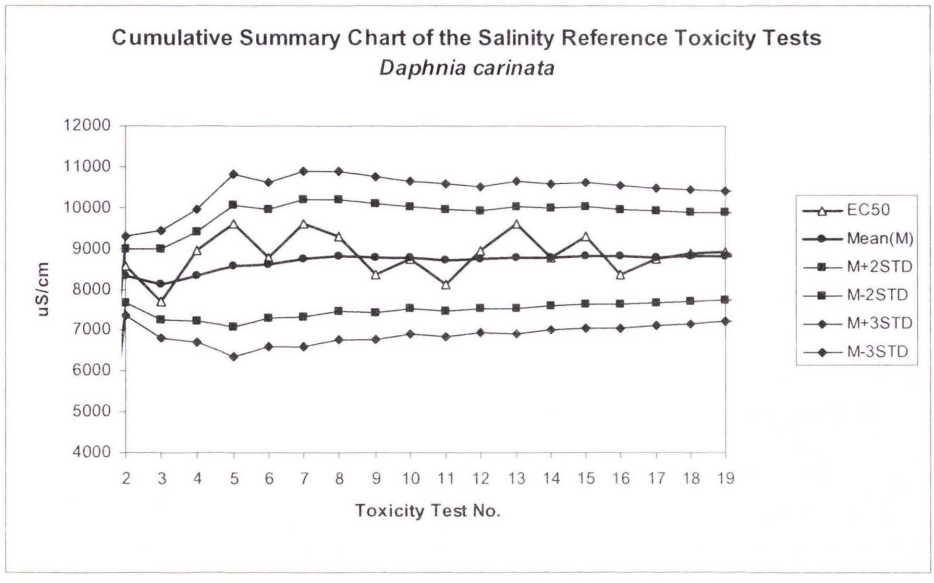

Figure 6.2: The cumulative summary chart for the salinity reference toxicity tests for *D. carinata.* (The actual salinity represented by hollow triangles, cumulative mean salinity represented by filled circles, twice the standard deviations represented by filled squares).

6.4.2. Toxicity experiments

6.4.2.1 Group 1 experiments

The EC50 values from the three tests conducted for each pesticide, together with the mean and the mean EC50 values for nineteen reference toxicant tests conducted with salinity are presented in Table 6.5.

Table 6.5: Concentrations of atrazine, chlorpyrifos, molinate and salinity that caused a 50% immobilisation of *D. carinata* (EC50) and the corresponding 95% confidence limits from valid toxicity tests. The mean and standard errors of these for the pesticides are based on three tests, while for salinity the mean is that for 19 toxicity tests.

Toxicant	Toxicity test number			Mean EC50
	1	2	3	
Salinity (µS/cm)	Mean from 19 toxicity experiments			8791 ± 524
Atrazine (mg/L)	42.5 (39.3 – 45.9)	41.2 (36.1 – 46.9)	43.7 (39.9 – 47.8)	42.4 ± 1.3
Molinate (mg/L)	25.4 23.2 – 27.0)	25.7 (22.7 – 29.0)	24.3 (21.7 – 27.3)	25.1 ± 0.7
Chlorpyrifos (µg/L)	0.212 (0.192– 0.234)	0.219 (0.198–0.240)	0.207 (0.190–0.226)	0.213 ± 0.01

There was little variability among the EC50 values for the three toxicity tests for each pesticide.

Based on the USEPA toxicant classification scheme (USEPA 2006), the results indicate that chlorpyrifos was very highly toxic while atrazine and molinate were slightly toxic to *D. carinata*.

For comparison of toxicities of individual chemicals, the EC50 values were expressed in moles/L (Table 6.6) (Warne and Schifko 1999). The order of the toxicity of pesticides was chlorpyrifos > molinate > atrazine.

Table 6.6: EC50 values of atrazine, molinate and chlorpyrifos to *D. carinata* expressed in moles/L.

Toxicant	EC50 (moles/L)
Atrazine	1.97×10^{-4}
Molinate	1.34×10^{-4}
Chlorpyrifos	6.07×10^{-10}

6.4.2.2 Group 2 experiments

The results of types of toxic interactions and how these vary with increasing salinity for mixtures of individual pesticides and salinity are presented in Figure 6.4. The various EC50 values of these mixtures are presented in Table 6.7.

For the 0.5 times the EC50 value of atrazine and salinity mixture, no toxicity was observed up to 0.5 TU. As the TU values of the mixture increased, toxicity conformed firstly to additivity and then became synergistic (Figure 6.4A). The EC50 value was 0.93 TU (Table 6.7), indicating at that concentration that the mixture conformed to additivity. In the mixture of the EC50 value of atrazine and salinity (Figure 6.4B), the toxicity conformed to additivity. The EC50 value for this mixture could not be calculated, as 50% immobilisation occurred before the first treatment (i.e., 1 TU).

The toxicity of the 0.5 times the EC50 value of the molinate and salinity mixture conformed to antagonism, except that at a TU of greater than 1.5 the toxicity conformed to additivity (Figure 6.4C). The EC50 value of this mixture was 1.47 TU (Table 6.7), conforming to antagonism. The toxicity of the EC50 value of the

molinate and salinity (Treatment 2 is the molinate only treatment) mixture Treatment 6 conformed to additivity (2.14 TU) while Treatments 2, 3, 4 and 5 conformed to antagonism. The EC50 value of this mixture was 1.90 TU (Table 6.7), conforming to antagonism. The toxicity of the EC50 values of the chlorpyrifos and salinity mixture (Figure 6.4E) conformed to antagonism (e.g. the EC50 value was 1.92 TU) but as the concentration of the mixture increased beyond 2 TU, the toxicity conformed to additivity. In contrast, the toxicity of the 1.5 times EC50 value of chlorpyrifos and salinity (Treatment 1 is the chlorpyrifos only treatment) Treatments 2 and 6 conformed to additivity while Treatments 3, 4 and 5 conformed to antagonism (Figure 6.4.F).

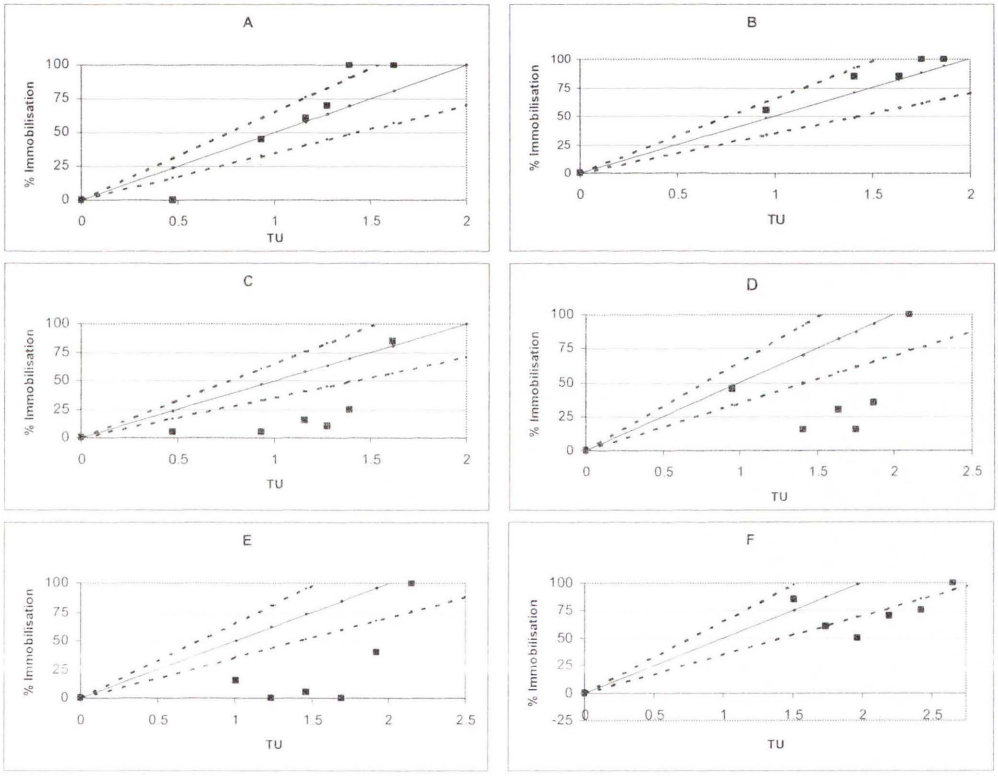

Figure 6.3: The toxicity of mixtures of individual pesticides with varying salinities to *Daphnia carinata* cultured in water with a salinity of 200 µS/cm. A - 0.5 x EC50 of atrazine and salinity; B - EC50 of atrazine and salinity; C - 0.5 x EC50 of molinate and salinity; D - EC50 of molinate and salinity; E - EC50 of chlorpyrifos and salinity; F - 1.5 x EC50 of chlorpyrifos and salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

Table 6.7: The EC50 values of mixtures of individual pesticides and salinity, expressed as toxic units (TUs).

Mixture combinations	EC50 TU
Atrazine (0.5 x EC50) and salinity	0.93 (0.84 – 1.04)
Atrazine (EC50) and salinity	ND
Molinate (0.5 x EC50) and salinity	1.47 (1.41 – 1.53)
Molinate (EC50) and salinity	1.90 (1.83 – 1.98)
Chlorpyrifos (EC50) and salinity	1.92 (1.87 – 1.98)
Chlorpyrifos (1.5 x EC50) and salinity	ND

ND – Could not be determined

6.4.2.3. Group 3 experiments.

The variation in toxicity of the equitoxic pesticide mixtures with salinity is presented in Figure 6.5 and their EC50 values are presented in Table 6.8. The toxicity of the atrazine and molinate mixture conformed to antagonism and then conformed to additivity in Treatment 4 and with synergism in Treatment 5 where 95% immobilisation occurred (Figure 6.5A). The EC50 value for this mixture was 1.04 TU (i.e. it conformed to additivity) (Table 6.8). The variation in toxicity for the atrazine and chlorpyrifos mixture was similar to that for atrazine and molinate, but the deviations from additivity to antagonism were more marked (Figure 6.5B). The EC50 value for this mixture was 1.19 TU which conformed to additivity (Table 6.8). The molinate and chlorpyrifos mixture conformed to additivity, apart from 0.5 and 1 TU (Treatments 2 and 3) where the toxicity conformed to antagonism (Figure 6.5C) and the EC50 value was 1.15 TU conforming to additivity (Table 6.8). In the mixture of all three pesticides, there was no toxic response up to 0.5 TU, but as the concentration increased the mixture firstly conformed to additivity and then synergism (Figure 6.5D). The EC50 value for this mixture was 0.92 TU again conforming to additivity (Table 6.8).

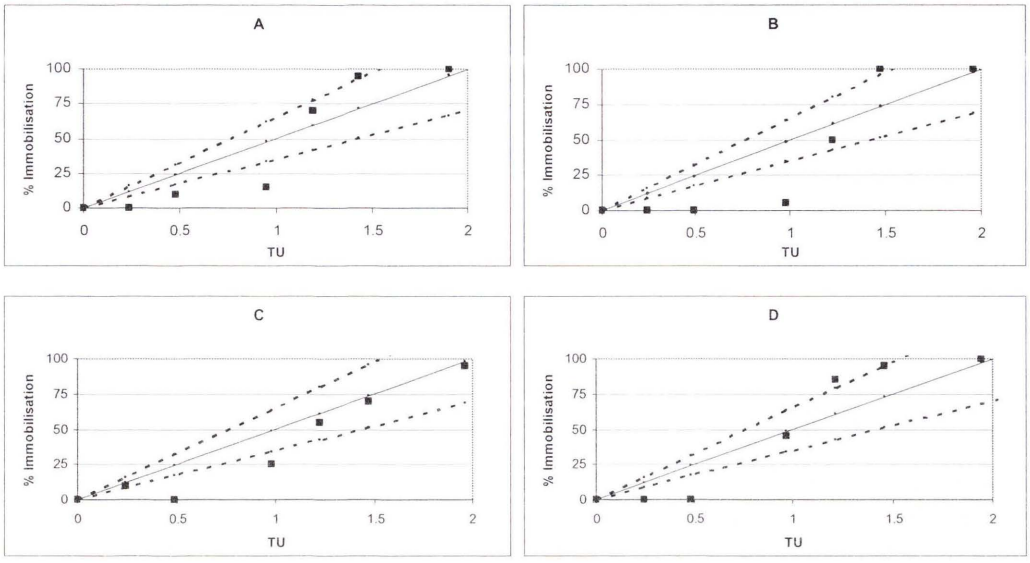

Figure 6.4: The toxicity of equitoxic mixtures of pesticides to *D. carinata* cultured in water with a salinity of 200 µS/cm. A - Atrazine and molinate; B - Atrazine and chlorpyrifos; C - Molinate and chlorpyrifos, D - Atrazine, molinate and chlorpyrifos. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

Table 6.8: The EC50 values of equitoxic mixtures of three pesticides, expressed as toxic units (TUs)

Toxic Combinations	EC50 TU
Atrazine and molinate	1.04 (0.91 – 1.19)
Atrazine and chlorpyrifos	1.19 (1.11 – 1.27)
Molinate and chlorpyrifos	1.15 (1.01 – 1.31)
Atrazine, molinate, chlorpyrifos	0.92 (0.83 – 1.03)

6.4.2.4. Group 4 experiments.

Toxicity relationships of mixtures of pesticides and salinity are presented in Figure 6.6, while the toxicity of the mixtures (in terms of TUs) at the EC50 is presented in Table 6.9.

The toxicity relationship for the mixture of atrazine and molinate with salinity conformed to additivity (Figure 6.6A). The EC50 value for this combination was 1.06

(Table 6.9). The relationship for the mixture of atrazine and chlorpyrifos with salinity also conformed to additivity (Figure 6.6B). The EC50 value obtained was 1.16 TU (Table 6.9). The toxicity relationship for the mixture of molinate, chlorpyrifos and salinity was antagonistic in Treatment 2 where the EC50 value was 1.41 and then with increasing mixture concentration moved towards additivity. The relationship of the complex pesticide mixture of atrazine, molinate, chlorpyrifos and salinity was additive and then became synergistic above 1.2 TU (Figure 6.6D). The expected toxicity response (50% immobilisation) occurred in the pesticide-alone treatment (TU1).

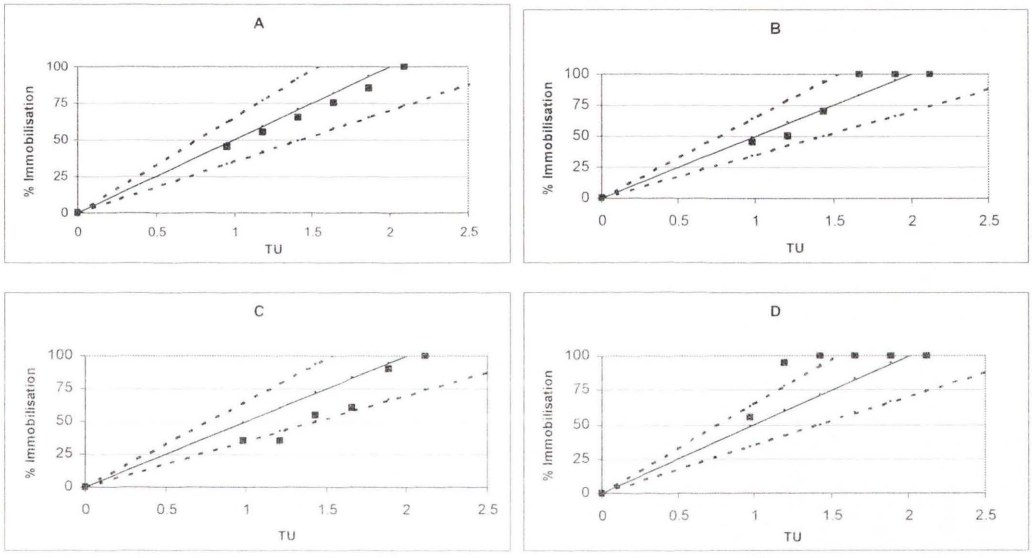

Figure 6.5: The toxicity of mixtures of pesticides with varying concentrations of salinities to *D. carinata* cultured in water with salinity of 200 µS/cm (the toxic contribution from the combination of pesticides is equal to 1TU). A - Atrazine/molinate/salinity; B - Atrazine/chlorpyrifos/salinity; C - Molinate/chlorpyrifos/salinity ; D - Atrazine/molinate/chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

Table 6.9: EC50 values of mixtures of pesticides with salinity expressed as toxic units (TUs).

Mixture Combinations	EC50 TU
Atrazine, molinate and salinity	1.06 (0.76 – 1.49)
Atrazine, chlorpyrifos and salinity	1.16 (0.78 – 1.72)
Molinate, chlorpyrifos and salinity	1.41 (1.21 – 1.64)
Atrazine, molinate, chlorpyrifos and salinity	ND

ND – Could not be determined

6.4.2.5 Occurrence of toxicity relationships

The types and frequency of occurrence of toxicity relationships of the different mixture combinations are presented in Table 6.10.

Table 6.10: Types of toxicity relationships observed in mixture combinations and their frequency of occurrence.

Toxicity relationship	Frequency of occurrence
Additive	3
Changed from antagonism to additivity*	4
Changed from additivity to antagonism and back to additivity*	2
Changed from additivity to synergism*	1
Changed from antagonism to additivity and then to synergism*	4
Total	14

* - Changes in toxicity relationship occurred with increase in concentrations of mixtures.

Out of 14 toxicity mixtures three were additive, four combinations changed from antagonism to additivity, two changed from additivity to antagonism and back to additivity, while the remaining five relationships were synergistic at least at high concentrations.

There were 69 different combinations of mixtures in the total of 14 mixtures studied. Table 6.11 summarises the number and percent occurrence of the types of toxicity relationships in these combinations.

Table 6.11: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos.

Toxicity relationship	Number of occurrence	% Occurrence
Additive	34	49.3
Antagonistic	28	40.6
Synergistic	7	10.1

The majority of the toxicity relationships were additive (approx. 50%) and antagonistic and synergistic combinations were approximately 41% and 10%, respectively.

6.5. Discussion

As the EC50 value of each test is within two standard deviations of the long-term mean EC50 value, it was considered that the relative sensitivities of the test organisms were stable over time with no change in the sensitivity to salt during the period of the study. This is important as it permits the toxicity of tests conducted at different times to be directly compared with any differences being attributable to the various treatments.

The mean acute EC50 (immobilisation) value for salinity in the present study was 8,800 µS/cm. Working on the toxicity of sodium chloride and a commercially available salinity (Ocean Nature) to *D. carinata*, Kefford et al. (2004) recorded EC50 values of 11,000 and 4,500 µS/cm, respectively. These large differences in EC50 values show that the ionic composition of the salt plays an important role in determining its toxicity. The differences in EC50 values obtained by the current study and those of Kefford et al. (2004) could be explained by the different ionic compositions. Another reason for the observed differences in toxicity could be that there were differences in genetic characteristics in the *D. carinata* populations used.

The salinities in Australian inland waters exhibit a vast range (Pinder et al. 2005). Table 6.12 shows the variations in salinity in different water bodies in the Western Australian wheat belt.

Table 6.12: Salinities of water bodies in Western Australian wheat belt (Source: Pinder et al. 2005).

No. of sites	Salinity (μS/cm)
86	< 4,300
46	4,300 – 14,000
69	14,000 – 140,000

In many Western Australian water bodies the salinity exceeds the mean acute toxicity of salinity to *D. carinata* estimated by the current study (Table 6.5). The chronic and multiple generation impacts on *D. carinata* appear even at lower salinities (i.e., 5500 μS/cm, (see Chapter 5) and thus such effects are likely to occur in the real world. It is important to have information on the acute toxicities of salinity to the organism under consideration in order to understand the concentrations that cause acute lethal effects. Aqueous salinities less than or equal to the internal salinities of organisms do not cause toxic effects (Arner and Koivisto 1993). This is mainly due to the ability of cladocerans to osmoregulate their body fluids in relation to the external medium (Aladin and Potts 1995). Aqueous concentrations above that threshold can affect life-history traits and populations (see Chapter 5). Death can occur when the aqueous concentrations are sufficiently larger than the threshold as shown by the EC50 values for salinity in the present study.

Based on EC50 values for *D. carinata*, chlorpyrifos was the most toxic pesticide, followed in order of decreasing toxicity by molinate and finally atrazine. This is not surprising, since atrazine and molinate are herbicides and cladocerans do not have the target site with which these herbicides interact. Chlorpyrifos inhibits acetyl cholinesterase in animals, including *D. carinata*, and thereby causes death at very low concentrations. The inhibition of acetylcholinesterase causes the buildup of acetylcholine at choline receptors, which leads to continual nerve stimulation (Giesy et al. 1999). More importantly the metabolic products of chlorpyrifos are more toxic and the high toxicity is caused by the formation of chlorpyrifos oxon by oxidative desulfuration (Eisler 2000; Giesy et al. 1999). Factors influencing the toxicity of chlorpyrifos include metabolic rate, the number of target sites available for chlorpyrifos metabolism to chlorpyrifos oxon (Chambers and Carr 1995), organism surface area, and lifestage (El-Merhibi et al. 2004).

The EC50 values for the three pesticides are consistent with values obtained in other studies. Munn and Gilliom (2001) report EC50 values for atrazine to *Daphnia magna* to be between 6.9 and 115 mg/L, with a median value of 60.9 mg/L. The reported EC50 values for molinate ranged from 4.7 to 24.0 mg/L, with a median value of 19.4 mg/L, while the EC50 values for chlorpyrifos varied from 0.10 to 1.70 μg/L, with a median of 0.90 μg/L. The EC50 (immobilisation) values recorded for *D. carinata* to molinate by Phyu et al. (2004) ranged from 18.3 to 33.6 mg/L are in good agreement with the values of the present study. The EC50 values for atrazine for *D. carinata* recorded by Phyu et al. (2004) were 22.4 to 26.7 mg/L which was less than the values of the present study.

Implications of synergism in mixture toxicities at low concentrations

Before discussing the results of the mixture toxicity tests, it is important to explain a feature of some of the results – the tendency at high TU values for the toxicity of mixtures to change from conforming to synergism to conforming to additivity. This is a result of the nature of the experimental design of the toxicity tests rather than an actual change in the type of toxicity. This can best be explained by referring to a plot of a mixture toxicity test – in this case the plot of the % immobilisation of *D. carinata* against the total TU in a mixture of atrazine/molinate/chlorpyrifos and salinity (Figure 6.6).

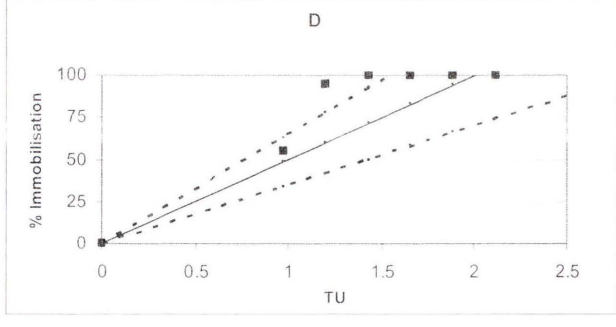

Figure 6.6: The variation in the toxicity of a mixture of atrazine, chlorpyrifos, molinate and salinity with increasing salinity (extracted from Figure 6.5). The TU values are for the entire mixture. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. 100% immobilisation occurred in the treatment of 1.43 TU.

At 1 TU, which is the pesticide-only (mixture of all three) treatment, the toxicity of the mixture is consistent with additivity but with increasing salinity it rapidly changed to being consistent with synergism, with nearly 100% of *D. carinata* being immobilised. At Treatment 2 (above 1 TU), 100% of the organisms were immobilised. Further increases in the salinity could possibly immobilise more cladocerans but in practice this is not possible as the number of cladocerans per treatment is controlled. Thus in this example, further increases in salinity will continue to cause 100% immobilisation and at 2 TU (where, based on additivity one would expect 100% immobilisation) 100% immobilisation is also achieved. However, for the reasons stated earlier it is not possible to know whether toxicity has reverted to conforming to additivity or whether it is a function of the experimental design of the toxicity test. In this example, where there are a series of concentrations of the mixture with 100% immobilisation, the second explanation for the apparent conformity with additivity is the most likely explanation. This situation only applies if lower concentrations of the mixture are synergistic – it does not apply if the toxicity conforms to antagonism. Thus, reliable interpretation of the results of mixtures is only possible up to the point where 100% immobilisation is first achieved.

In terms of the toxicity of mixtures of the individual pesticides and salinity, two main types of relationships were observed. Atrazine and salinity combinations conformed to additivity and changed from additivity to synergism (at 0.5 times EC50 value of atrazine and salinity), whereas for the molinate and salinity and the chlorpyrifos and salinity combinations the toxicities conformed to antagonism and additivity. As salinity increases the osmoregulatory mechanism changes in order to maintain the internal body and haemolymph salinities of the test organisms (Aladin and Potts 1995; Pinder et al. 2005). Associated with changes in the osmoregulatory mechanisms, there could also be changes in selective permeability of the membrane, to prevent entry of chemicals into body tissue (Aladin and Potts 1995). This could be the reason for the reduction in toxicity of molinate and chlorpyrifos with increasing salinity to a certain level (Figure 6.5). The toxicity of atrazine and salinity conformed to additivity, and with synergism at high concentrations. This could be due to the changes in the osmoregulatory mechanism not affecting the permeability of atrazine or that

movement of atrazine to its target site in cladocerans is independent of the permeability of the membrane to this herbicide.

The toxicity of the equitoxic mixtures of molinate and chlorpyrifos conformed to additivity in Treatment 2 and with antagonism in Treatments 3 and 4, and finally became additive as the concentrations increased. The remaining three equitoxic mixtures conformed to antagonism at concentrations below 1 TU and then with additivity and finally sometimes with synergism at the higher concentrations. Even though many studies have concluded that additivity occurs with compounds with similar modes of action (Altenburger et al. 1990), we observed that toxicity consistent with additivity can occur in mixtures of chemicals with different modes of action. The toxicity relationship of atrazine and chlorpyrifos in the present study was consistent with antagonism below 1 TU, with additivity (above 1 TU) and synergism at around 1.5 TU. This concurs with the findings of Beldin and Lydy (2000), Pape-Lidstrom and Lydy (1997), who worked on *Chironomus tentans* and observed synergistic effects for the same chemicals. A possible reason is the increase in the rate of biotransformation of chlorpyrifos to more toxic metabolites (e.g., chlorpyrifos-*O*-analog) in the presence of atrazine (Belden and Lydy 2000; Londoño et al. 2004; Pape-Lindstrom and Lydy 1997) related this phenomenon to the induction of cytochrome P450 in the presence of atrazine and potentiation of the degradation of chlorpyrifos to more toxic metabolites.

The toxicities of the complex pesticide mixtures atrazine / molinate/ salinity and atrazine / chlorpyrifos / salinity were consistent with additivity. The molinate / chlorpyrifos / salinity mixture, which was consistent with additivity in Treatment 2, became additive at higher concentrations. The atrazine/chlorpyrifos/molinate/salinity mixture was consistent with additivity in Treatment 1 and with synergism in the next two treatments. At subsequent treatments the toxicity relationship could not be determined as 100% mortality was reached (refer to section 6.4.2.2). The antagonistic effect at lower concentrations in the molinate/chlorpyrifos/salinity mixture could be due to the changes in the cladoceran's osmoregulatory mechanism with increasing salinity. However, at higher concentrations this mechanism appeared to have failed and relationship became additive.

Given the diversity of possible mechanisms of actions (MeOAs) amongst chemicals in mixtures likely to be experienced in the environment, it is unlikely that the requirements of the concentration addition (CA) model (that components of the mixture have the same MeOA) will be met. In the present study, the three pesticides tested have three different MeOAs and salinity has a non-specific mode of action. However, a number of laboratory and field-based studies has shown that the use of concentration addition is a realistic worst case scenario and, hence, it is appropriate to use this model in the first tier of the proposed framework of risk assessment. The CA model is conservative in two ways. First, Deneer (2000), Faust et al. (1994), Warne and Hawker (1995) and Ross and Warne (1997) have found that approximately 10 – 30% of mixtures (irrespective of the type of chemical) were antagonistic or synergistic, with each type of joint action being equally frequent. Thus by assuming CA, the toxicity of 85 to 95% of mixtures would be estimated accurately or overestimated and only 5 to 15% of mixtures would have their toxicities underestimated. Second, Faust et al. (1994); Backhaus et al. (2000a and b), Dyer et al. (2000), Junghans et al. (2006) and Chevre et al. (2006) found that CA overestimated the effects and yielded slightly higher estimates of the toxicity of mixtures than independent action (IA) where the chemicals had different MeOAs. As the CA model is likely to over-predict the toxicity of most mixtures its use in Tier 1 should be environmentally protective.

In the present study, 14 combinations of mixtures were used (Table 6.10). Among these, two combinations changed from additivity to antagonism and back to additivity. The rest of the combinations were either additive (three combinations), changed from antagonism to additivity (four), changed from antagonism to additivity and to synergism (four), or changed from additivity to synergism (one). Of the 14 combinations, five were synergistic and produced toxicities greater than the CA model predicted at least at higher concentrations (Table 6.10).

A total of 69 mixture combinations was tested. Of this 40.6% of combinations conformed to antagonism, 49.3% conformed to additivity and 10.1% conformed to synergism (Table 6.11). Deneer (2000), Faust et al. (1994), Warne and Hawker (1995) and Ross and Warne (1997) found that approximately 5 – 15% of mixtures

(irrespective of the type of chemical) were antagonistic. However, results of the present study indicate that the percentage occurrence of antagonism did not concur with that of the above studies. The results showed that approximately 90% of the mixture combinations (additive and antagonistic) would be estimated accurately or overestimated by the CA model. Only 10% would have their toxicity underestimated.

Thus, mixtures exerting these types of toxicities should be given due consideration in formulating risk assessments. In order to determine the requirements for further assessment, baseline information on toxicity relationships is necessary and the present study provides such information on all possible combinations of mixture toxicities of salinity, atrazine, molinate and chlorpyrifos.

6.6. Conclusions

The acute toxicities of pesticides to *D. carinata* in descending order were, chlorpyrifos (EC50 - 0.213 µg/L), molinate (EC50 - 25.1 mg/L) and atrazine (EC50 - 42.4 mg/L). Salinity caused 50% immobilisation of *D. carinata* at 8,800 µS/cm. The toxicities of mixtures of pesticides and salinity produced results that were consistent with approximately, 40% being antagonistic, 50% additive, and 10% synergistic. The toxicity relationships of some of the mixtures varied with concentration. These variations may be related to changes in osmoregulatory activity. The use of the concentration addition model of mixture toxicity would provide protective estimates for approximately 90% of the mixtures tested.

Chapter 7

Acute toxicity of salinity and the pesticides, atrazine, molinate and chlorpyrifos individually and as mixtures to *Daphnia carinata* acclimatised at elevated salinities

7.1. Abstract

Aquatic organisms inhabiting salinity-affected inland water bodies experience the effects of salinity throughout their life cycle and over multiple generations. Salinity and pesticide pollution often occur in the same locations due to the fact that irrigation can enhance salinisation of the environment. It is therefore important to determine if exposure to elevated salinity will modify the susceptibility of aquatic organisms exposed concurrently to salinity and pesticides.

The freshwater cladoceran, *Daphnia carinata,* was acclimatised in water of two salinities (i.e., 200 and 6300 μS/cm) over 13 generations. The salinity-acclimatised neonates were then subjected to a series of acute tests designed to determine the median immobilisation salinity or concentration (EC50 (immob.)) of: salinity, atrazine, molinate and chlorpyrifos; mixtures of each pesticide and elevated salinity; mixtures of the pesticides, and mixtures of the pesticides and elevated salinity.

The EC50 values of individual toxicants and each mixture for *D. carinata* cultured at 200 and 6300 μS/cm were compared. The only cases where there were significant ($p \leq 0.05$) changes in toxicity were for salinity and for the mixture of atrazine (0.5 x EC50) and salinity where in both cases the elevated salinity acclimated cultures were less sensitive. Among the mixture combinations for the elevated salinity acclimated cultures, 52% conformed to additivity while approximately 37% conformed to antagonism and 11% conformed to synergism. The acclimation to elevated salinity led to a slight decrease in mixtures that conformed to antagonism and a commensurate increase in mixtures that conformed to additivity. Overall, the results indicate that the

sensitivity of *D. carinata* cultured at normal (i.e., 200 µS/cm) and elevated salinity (6300 µS/cm) behaved in the same manner in response to individual toxicants of atrazine, molinate, chlorpyrifos and salinity, and their mixtures.

7.2. Introduction

The salinisation of freshwater environments is occurring in many parts of the world (Jolly et al. 2001; Williams 1987). In Australia, the process has been occurring for a prolonged period and both the area of land affected and the severity of the effects are likely to increase (Jolly et al. 2001; National Land & Water Resources Audit 2001). Jolly et al. (2001) have shown that the mean rate of increase in salinity in the Murray-Darling basin (Australia's largest river catchment) at present is 4.37 µS/cm/year with a minimum of -6.9 to a maximum of 139.5 µS/cm/year (mean for 87 monitoring stations).

The acute effects of salinisation on aquatic organisms have been fairly well studied and documented in Australia and internationally (Halse et al. 1998; Hart et al. 1991; Hart et al. 2003; James et al. 2003; Kefford 1998; Kefford et al. 2002; Kefford et al. 2004; Mohammed and Agard 2007). These have predominantly focussed on laboratory-based data (Mohammed and Agard 2007), although recent work by Kefford et al. (2004) has correlated laboratory-based results with field-based data on the occurrence of invertebrates in salinity affected environments to provide a more holistic view of the potential effects of elevated salinity on freshwater aquatic organisms.

A major limitation of the vast majority of the available toxicity data on salinity is that it is based on acute exposure to salinity. In aquatic environments that are suffering long-term increases in salinity, organisms are likely to be exposed to elevated salinity over their entire lives and indeed over many generations. The areas in Australia suffering from salinisation are also often contaminated with pesticides. It is therefore likely that aquatic organisms experiencing elevated salinity will concurrently be

exposed to individual pesticides and their mixtures. There is limited information on the toxic effects of elevated salinity and pesticides, let alone information on the potential interaction of exposure to elevated salinity levels and other toxicants.

There are a number of potential scenarios of what could happen to organisms exposed for multiple generations to elevated salinity and pesticides. They could become more sensitive or develop tolerance to salinity or retain their original sensitivity. For example, cladocerans exposed to metals (Gulati et al. 1988; LeBlanc 1985) and organics (Baldwin et al. 1997) have been shown to develop tolerance to these toxicants. In contrast, exposure of several generations of *Daphnia magna* to the pesticides diazinon (Sanchez et al. 2000) and tetradifon (Villarroel et al. 2000b) resulted in a decrease in tolerance over successive generations. Each of these modifications in sensitivity could in turn lead to sensitivity to other toxicants increasing, decreasing or remaining the same. According to the "metabolic cost" hypothesis (Calow and Sibly 1990) development of tolerance will increase energy consumption resulting ultimately in adverse effects on reproduction and growth. Therefore, cladocerans more tolerant of salinity may have less energy available to respond to other toxicants. Tolerance to other toxicants could decrease if they have the same mechanism of action as salinity or the same detoxifying or excretion mechanism. Tolerance to other toxicants could remain unaffected if the shift in tolerance did not incur any metabolic cost or if the organism's means of detoxifying or excreting them was not the same as that for salinity.

Information on chronic or multigenerational exposure to metals (Guan and Wang 2006; Vogt et al. 2007) and organic chemicals (Rose et al. 2002b), pesticides (Fernandez-Casalderrey et al. 1995; Ferrando et al. 1996; Sanchez et al. 2004; Villarroel et al. 2000a; Villarroel et al. 2000b; Zalizniak and Nugegoda 2006) provides useful insights on how animals respond to toxicants in such situations. As stated above, organisms can be resistant or more sensitive due to disruption of their defence systems (Muyssen and Janssen 2002) or there may not be visible effects in exposed animals, which may be due to the nature of the toxic effects of the toxicant under consideration. Thus it is important to understand what kinds of effects are

created by salinity on the sensitivity of animals to multiple mixtures of stressors found in the environment.

The present study, therefore, aims to determine the acute toxicity of salinity, atrazine, molinate and chlorpyrifos; mixtures of each pesticide and elevated salinity; mixtures of the pesticides; and mixtures of the pesticides and elevated salinity to *D. carinata* acclimatised to elevated salinity and to ascertain if acclimation to elevated salinity affected their sensitivity to the other toxicants.

7.3. Materials and Methods

7.3.1. Test species

The freshwater cladoceran, *Daphnia carinata* is representative of freshwater zooplankton and is commonly used for toxicity testing (Chandini 1989, Van Dam et al. 1995, Zalizniak and Nugegoda 2004, Phyu et al. 2004). The desirable features of *D. carinata* are as follows: it is easy to maintain and culture; has a relatively short life cycle (i.e., they breed after approximately 10 days and live for approximately 60 - 80 days); it represents the primary consumer trophic level of and as such is linked to the higher levels of food webs via zooplanktivorous fish. The *D. carinata* populations used in the present study were obtained from the Centre for Ecotoxicology, NSW Department of Environment and Climate Change (NSW DECC), Lidcombe, NSW, Australia, and were originally sourced from the NSW Fisheries Research Station at Narrandera, NSW, Australia (Moreno Julli, NSW DECC, *pers. comm.*).

7.3.2. Toxicants

The herbicides atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1, 3, 5-triazine - 2, 4 - diamine; CAS no-1912-24-9) and molinate (s-ethyl *N*, *N* -hexamethylenethio carbamate; CAS no-2212-67-1), and the organophosphorus insecticide chlorpyrifos (O,O-Diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CAS No. 2921-88-2), were selected, as they are widely used in Australian agriculture (Bowmer et al. 1998)..

The pesticides were all reagent-grade technical chemicals (≥ 97% purity). Sea water, which is of the same ionic composition as Australian inland salt (Bayly and Williams 1972), provided the different salinities needed for the study.

Stock and working solutions of pesticides were prepared in analytical grade (99% purity) acetone as the carrier solvent. The stock solutions were stored in a freezer at -4°C and all working stock solutions were made immediately prior to use.

7.3.3. *D. carinata* cultures

The culturing of *D. carinata* was carried out according to the methods specified in the Methods Manual (2005) of the Ecotoxicology Section of the NSW DECC. Details of the culture techniques are described in Chapter 5.

Salinity acclimatisation of cladocerans was achieved by culturing them at 6300 µS/cm, using the same culture techniques described in Chapter 5. The salinity level 6300 µS/cm was based on the results of the acute toxicity tests. Since there should be viable survivors in order to maintain the cultures it was decided not to exceed 40% immobilisation at upper concentration. Based on the results of salinity reference toxicity tests (on average), 35% immobilisation occurred at the salinity of 6300 µS/cm and this was therefore selected as the elevated salinity level. The high salinity cultures (6300 µS/cm) were maintained over a year before using them for the toxicity tests. The salinity acclimatised cladocerans were subjected to individual acute toxicity testing with salinity and three pesticides before proceeding with the mixture toxicity studies.

7.3.4. Experimental design

The toxicity experiments were conducted in four groups. An overall description of the experimental design of these four groups is provided below.

7.3.4.1 Group 1 experiments

This set of experiments determined the acute toxicities for salinity, atrazine, molinate and chlorpyrifos, each tested separately to the cladoceran. Each experiment consisted of a control and five treatments of increasing concentrations of a pesticide or salinity. Based on the results of these tests, the concentrations of individual chemicals and salinity to be used in the mixture tests were determined.

7.3.4.2. Group 2 experiments

This set of experiments determined the toxicity of mixtures of salinity with individual pesticides (i.e., salinity/atrazine, salinity/chlorpyrifos, and salinity/ molinate). The treatments in these experiments used a fixed concentration of the pesticide with increasing salinity (Table 7.1). The experiments were designed to test the following pesticide concentrations:

- 0.5 TU for atrazine
- 1 TU for atrazine
- 0.5 TU for molinate
- 1 TU for molinate
- 1 TU for chlorpyrifos
- 1.5 TU for chlorpyrifos.

The concentrations (expressed as toxic units (TUs)) that were determined for the experiments are presented in Table 7.1.

Table 7.1: Concentration of each treatment of each pesticide and salinity mixture expressed in toxic units (TUs) and the contributions to the treatment of salinity. The pesticide in each mixture had a fixed concentration (1 TU) while that of salinity increased.

Treatment	Toxic contribution from salinity	Concentration of mixtures (TUs)		
		pesticide present at 0.5 TU	pesticide present at 1 TU	pesticide present at 1.5 TU
Control	0	0	0	0
1	0	0.5	1	1.5
2	0.46	0.96	1.46	1.96
3	0.68	1.18	1.68	2.18
4	0.79	1.29	1.79	2.29
5	0.91	1.41	1.91	2.41
6	1.14	1.64	2.14	2.64

7.3.4.3. Group 3 experiments

This set of experiments consisted of mixtures of all possible combinations of the pesticides (i.e., atrazine/molinate, atrazine/chlorpyrifos, chlorpyrifos/molinate and atrazine/molinate/chlorpyrifos). Each experiment consisted of a control and six pesticide mixture treatments of increasing TUs (Table 7.2). The mixtures were equitoxic, i.e., each pesticide is present in the mixture at the same percentage of their EC50 values measured individually. Thus, in Treatment 1 of the atrazine/chlorpyrifos/molinate test where the total TU is 0.24, each pesticide would be present at a concentration that corresponds to 0.08 TU of their individual EC50 values.

Table 7.2: Concentration of each treatment of the equitoxic pesticide mixtures expressed as toxic units (TUs).

Treatment	Concentration of mixtures (Toxic Units, TUs)			
	atrazine/ molinate	atrazine/ chlorpyrifos	chlorpyrifos/ molinate	atrazine/ chlorpyrifos/ molinate
Control	0.00	0.00	0.00	0.00
1	0.24	0.25	0.24	0.24
2	0.48	0.49	0.49	0.49
3	0.95	0.98	0.98	0.97
4	1.19	1.23	1.22	1.21
5	1.43	1.47	1.47	1.46
6	1.91	1.96	1.96	1.94

7.3.4.4. Group 4 experiments

This set of experiments consisted of combinations of two or more pesticides with salinity (i.e., atrazine / molinate / salinity, atrazine / chlorpyrifos / salinity, molinate / chlorpyrifos / salinity, atrazine / molinate / chlorpyrifos / salinity). Each experiment consisted of a control and six increasing concentrations of the mixture. All treatments of each mixture contained a fixed concentration of pesticide (i.e. a total of 1 TU) but increasing amounts of salinity (Table 7.3). The pesticide component of each mixture was present as an equitoxic mixture – thus in binary mixtures each pesticide was present at 0.5 TU and in tertiary mixtures each pesticide was present at 0.33 TU. .

Table 7.3: Concentration of each treatment of each pesticides and salinity mixture as toxic units (TUs) and their contributions to the treatment of the salinity. The pesticide mixtures had fixed concentrations (1 TU) while that of salinity increased.

Treatment	Toxic contribution from salinity	Concentration of mixtures (TUs)			
		atrazine/ molinate/ salinity	atrazine/ chlorpyrifos/ salinity	chlorpyrifos / molinate/ salinity	atrazine/ molinate/ chlorpyrifos/ salinity
Control	0.00	0.00	0.00	0.00	0.00
1	0.00	0.95	0.98	0.98	0.97
2	0.46	1.18	1.21	1.21	1.20
3	0.68	1.41	1.44	1.44	1.43
4	0.79	1.64	1.67	1.67	1.66
5	0.91	1.87	1.90	1.89	1.89
6	1.14	2.10	2.12	2.12	2.11

7.3.5. Test method

The test method used was based on USEPA protocols (USEPA 2002). The method used differed from the USEPA methods in that an Australian cladoceran species *D. carinata* was used, animals were maintained in mass cultures, and only algae were provided as food. The high-salinity cladoceran cultures used for the tests were maintained at 6300 µS/cm and the required salinity of the culture media was achieved by mixing filtered aerated sea water with cladoceran water. Details of test methods are presented in Chapter 6.

Acute toxicity tests for mixtures were conducted using the same methods that were used for testing the toxicity of individual chemicals, except that the test solutions were different.

7.3.6. Evaluation of mixture toxicity - Toxic Units (TUs)

The toxicity of individual components in mixtures and the mixtures themselves were expressed as toxic units (TUs) using the method described by Brown (1968). Typically the TU is calculated using,

$$TU_i = \frac{C_i}{EC_{p_i}} \qquad (1)$$

where the subscript denotes the component 'i' of a mixture, while C_i is the aqueous concentration of component 'I' in the mixture and ECp_i is the aqueous concentration of the component acting individually, which will cause a given toxic effect (e.g., LC50, EC20). In the present study the term ECp_i was always the EC50 (immobilisation) for *D. carinata*. Thus the TU values of 0.5 and 1 for individual chemicals mean that they are present in a mixture at 0.5 times their EC50 and at their EC50 values, respectively.

The results of the mixture toxicity experiments were plotted as illustrated in Figure 7.1. The concentration of the mixture that causes a certain % of immobilisation is expressed as toxic units. The solid line is the additivity line, which links the points 0 TU, 0% immobilisation; 0.5 TU, 25% immobilisation; 1 TU, 50% immobilisation; and 2 TU, 100% immobilisation. As indicated in ECETOC (2001), less than 30% deviation from expected additivity is considered as conforming to additivity and more than 30% deviation conforming either to antagonism or synergism. The interpretations of mixture toxicity in the present study will be based on the above model. The two dashed lines (Figure 7.1) indicate the 30% deviation from additivity and mixtures that lie to the right of the lower dashed line are classified as antagonistic, while those that lie to the left of the upper dashed line are classified as synergistic. Mixtures that conform to concentration addition (CA) will lie in between the two dashed lines.

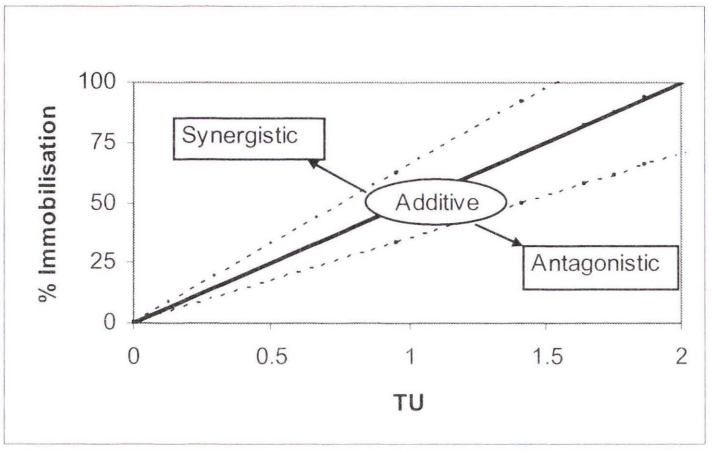

Figure 7.1: An example of the plots used to indicate the type of toxic interaction that occurs within the mixtures. The concentrations of the mixture (expressed in toxic units, TUs) that cause certain % immobilisation are plotted. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

7.3.7. Chemical analysis of test solutions

The concentrations of the pesticides were determined using the NSWEPA screening and extraction method for volatile and semi-volatile organic compounds. Chemical analysis of test solutions was conducted at the beginning and on completion of the test, to determine the loss of pesticides during the test. Details on the methods used for chemical analysis are described in Chapter 6.

7.3.8. Calculations and statistical analysis

The concentration of each pesticide and the salinity that immobilised 50% of the test organisms (EC50 immobilisation) and its 95% confidence limits were determined using the Trimmed Spearman-Karber method (Hamilton et al. 1977). The EC50 values of mixtures were also calculated using the same method except that the concentration of the mixtures was expressed as toxic units (Brown 1968). The statistical comparison of EC50 values of the corresponding high-salinity-acclimatised

and normal cladocerans were conducted using the standard error of the difference test (Sprague and Fogels 1977).

7.4. Results

The measured values pH (6.5 – 8.5), and electrical conductivity (1190 - 200 µS/cm), dissolved oxygen (4.0 – 7.5 mg/L) of all treatments of all the toxicity tests were within the acceptable limits.

7.4.1. Reference toxicant tests

The results of the reference toxicant tests (Cusum chart) for *D. carinata* cultured at 6300 µS/cm are presented in Figure 7.2 (see Chapter 6 for the Cusum chart for *D. carinata* cultured at 200 µS/cm). The mean EC50 values throughout the study were within two standard deviations of the long-term mean EC50 values (Figure 7.2) and therefore the results of concurrent toxicity tests (i.e., individual and mixture toxicity tests) were valid and comparable across tests. These results also indicate that the sea water (used for salinity treatments) quality is uniform and its toxicity did not vary between tests.

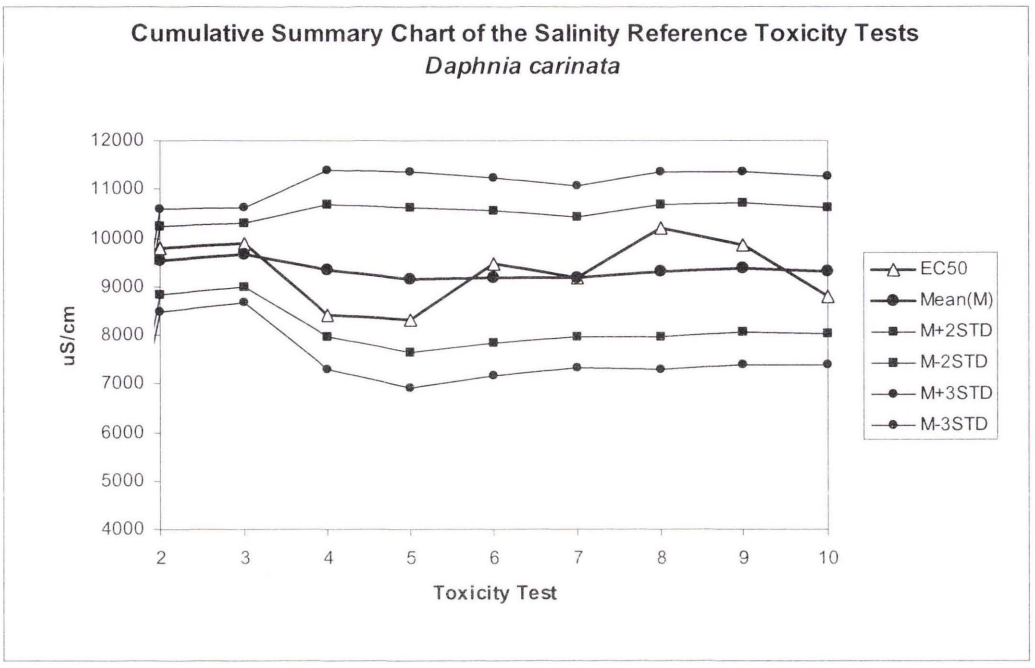

Figure 7.2: The cumulative summary chart for the salinity reference toxicity tests for *D. carinata* cultured in 6300 μS/cm medium. (The actual salinity is represented by hollow triangles, cumulative mean salinity is represented by filled circles, and twice the standard deviations are represented by filled squares).

7.4.2. Toxicity experiments

7.4.2.1 Group 1 experiments

The acute toxicity of salinity (EC50 value) to *D. carinata* cultured in water with salinities of 200 and 6300 μS/cm are presented in Table 7.4 (from the reference toxicity tests). The EC50 values of salinity-acclimatised *D. carinata* in four experiments were significantly ($p \leq 0.05$) higher than that of *D. carinata* cultured in normal cladoceran water. In one experiment, cladocerans cultured in normal cladoceran water (200 μS/cm) had a significantly ($p \leq 0.05$) higher EC50 value than the corresponding EC50 value of cladocerans cultured at 6300 μS/cm. No significant differences ($p > 0.05$) were observed in the five remaining experiments.

Table 7.4: Salinities that caused 50% immobilisation (i.e., EC50 values) of *D. carinata* cultured in normal cladoceran water (200 µS/cm) and elevated salinity (i.e. 6300 µS/cm).

Salinity EC50 (immobilisation) values for *D. carinata*	
Acclimatised at 200 µS/cm	**Acclimatised at 6300 µS/cm**
8117* (7524 – 8757)	9283* (8765 – 9831)
8944 (8533 – 9375)	9779 (8942 – 10695)
9604 (9029 – 10217)	9901 (9322 – 10516)
8791 (7389 – 10460)	8404 (7272 – 9712)
9289# (8867 – 9731)	8324# (7803 – 8880)
8349* (8067 – 8642)	9487* (9131 – 9855)
8738 (8218 - 9290)	9192 (8597 – 9828)
8881* (8148 – 9680)	10213* (9627 – 10834)
8921* (8641 – 9208)	9872* (9376 – 10394)
8499 (8119 – 8896)	8813 (8149 – 9530)

*** - The 6300 µS/cm salinity acclimatised *D. carinata* have EC50 values significantly larger than those acclimatised at 200 µS/cm ($p \leq 0.05$). # - The 200 µS/cm salinity acclimatised *D. carinata* have an EC50 value significantly larger than those acclimatised at 6300 µS/cm ($p \leq 0.05$).**

The individual toxicities of salinity, atrazine, molinate and chlorpyrifos are presented in Table 7.5.

Table 7.5: The salinity and concentration of atrazine, chlorpyrifos and molinate that caused 50% immobilisation of *D. carinata* cultured in water with salinities of 200, and 6300 µS/cm.

Toxicants EC50 (immobilisation) values for *D. carinata*		
Toxicant	**Acclimatised at 200 µS/cm**	**Acclimatised at 6300 µS/cm**
Salinity (mean salinity from ref. toxicity tests)	8791 ± 524	9309 ± 685*
Atrazine	42.5 (39.3 – 45.9)	41.5 (37.1 – 46.4)
Molinate	25.4 (23.2 – 27.0)	27.5 (24.0 – 31.5)
Chlorpyrifos	0.212 (0.192 – 0.234)	0.184 (0.166 – 0.204)

*** Significantly different at $p \leq 0.05$**

Based on the USEPA toxicant classification scheme (USEPA 2006), chlorpyrifos was very highly toxic while atrazine and molinate were slightly toxic to *D. carinata*.

For the comparison of toxicities of individual chemicals in both the normal and high salinity cultures, the EC50 values were expressed in moles/L (Table 7.6) (Warne and Schifko 1999). The order of toxicity for high salinity acclimatised cultures was chlorpyrifos > molinate > atrazine and the same pattern occurred in the cladocerans cultured at normal salinity (200 µS/cm).

Table 7.6: EC50 values of atrazine, molinate and chlorpyrifos to *D. carinata* expressed in moles/L.

Toxicant	EC50 (cultured at 200 µS/cm)	EC50 (cultured at 6300 µS/cm)
Atrazine (moles/L)	1.97×10^{-4}	1.92×10^{-4}
Molinate (moles/L)	1.34×10^{-4}	1.47×10^{-4}
Chlorpyrifos (moles/L)	6.07×10^{-10}	5.25×10^{-10}

The EC50 values of salinity to *D. carinata* cultured in 200 and 6300 µS/cm were significantly ($p \leq 0.05$) different with the cladoceran developing tolerance to salinity (i.e., had a higher EC50 value). There were no significant ($p > 0.05$) variations in the sensitivity of cladocerans cultured at 200 and 6300 µS/cm in the experiments for individual pesticides.

7.4.2.2. Group 2 experiments.

The toxicity of mixtures of each pesticide and salinity are presented in Figure 7.4. The toxicity of 0.5 x EC50 value of atrazine/salinity conformed to antagonism below 1 TU then with additivity and synergism at concentrations greater than 1 TU (Figure 7.4A). The toxicity of the EC50 value of the atrazine/salinity combination conformed to additivity (Figure 7.4B).

Both combinations with 0.5 times the EC50 value of molinate (Figure 7.4C) as well as the EC50 value of molinate with salinity (Figure 7.4D) conformed predominantly to antagonism but in some treatments to additivity. For chlorpyrifos and salinity (Figure 7.4E), 50% immobilisation occurred in the chlorpyrifos only treatment and

the mixtures conformed to antagonism in Treatments 2 – 5 and became additive in Treatment 6. In the 1.5 times the EC50 value of chlorpyrifos with salinity (Figure 7.4F) 100% immobilisation occurred at the chlorpyrifos only treatment. The toxicity relationship conformed to antagonism in Treatment 3 and to additivity in Treatments 4, 5 and 6.

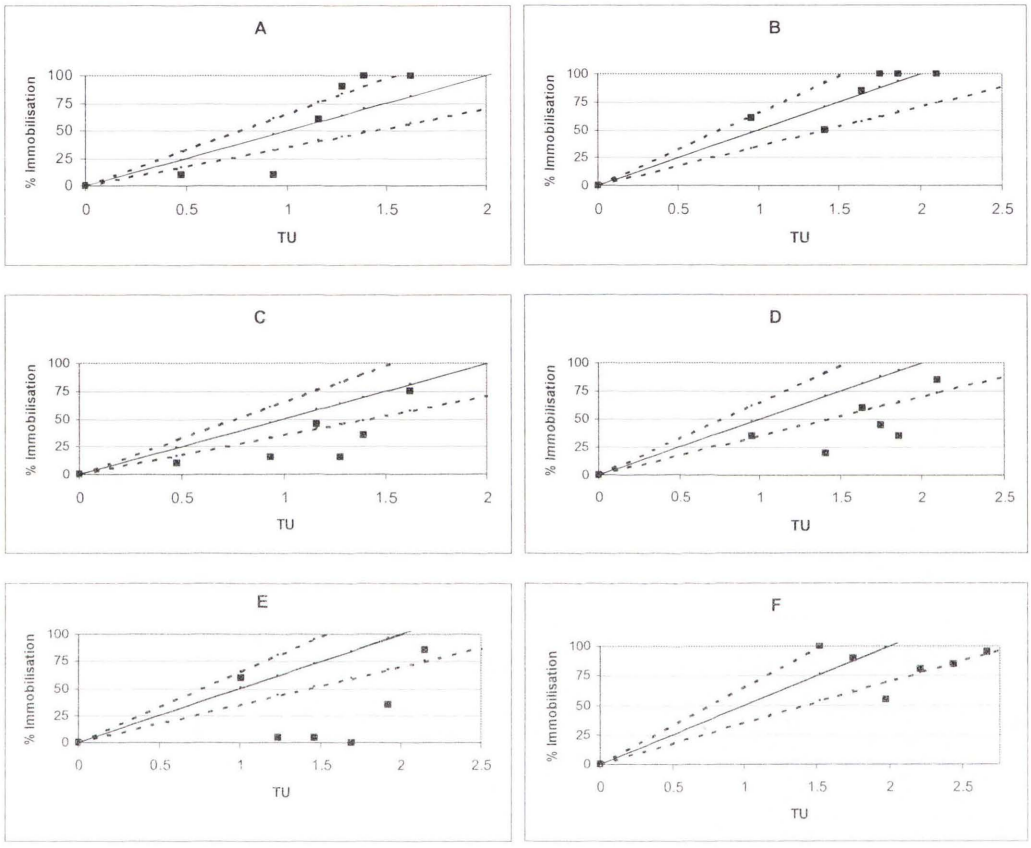

Figure 7.3: The toxicity of mixtures of individual pesticides with varying salinities to *Daphnia carinata* cultured in water with a salinity of 6300 µS/cm. A - 0.5 x EC50 of atrazine and salinity; B - EC50 of atrazine and salinity; C - 0.5 x EC50 of molinate and salinity; D - EC50 of molinate and salinity; E - EC50 of chlorpyrifos and salinity; F - 1.5 x EC50 of chlorpyrifos and salinity. The solid line indicates conformity with additivity while values to the left and right of the additivity line conform to synergism and antagonism respectively.

The toxicity at the EC50 of each of the above mixtures determined using *D. carinata* cultured in 200 and 6300 µS/cm water are presented in Table 7.7. The toxicity of the 0.5 times the EC50 value of atrazine and salinity mixture to *D. carinata* cultured in 6300 µS/cm water was significantly ($p \leq 0.05$) lower (i.e., the EC50 values were

larger) than the cladocerans cultured in 200 µS/cm water. None of the EC50 values of the other mixtures of pesticides and salinity were significantly different ($p > 0.05$) for cladocerans cultured in 200 and 6300 µS/cm water.

Table 7.7: The sum of the toxic units (TU) of mixtures of individual pesticides and salinity that caused 50% immobilisation of *Daphnia carinata*.

Mixtures	Mixture EC50 (immobilisation) values for *D. carinata* (TUs)	
	Cultured at 200 µS/cm	Cultured at 6300 µS/cm
Atrazine 0.5 x EC50 and salinity	0.93* (0.84 – 1.04)	1.10* (1.05 – 1.16)
Atrazine EC50 and salinity	ND	ND
Molinate 0.5 x EC50 and salinity	1.47 (1.41 – 1.53)	1.44 (1.32 – 1.56)
Molinate EC50 and salinity	1.9 (1.83 – 1.98)	1.75 (1.61 - 1.90)
Chlorpyrifos EC50 and salinity	1.92 (1.87 – 1.98)	1.96 (1.88 – 2.05)
Chlorpyrifos 1.5 x EC50 and salinity	ND	ND

*** – Mixture EC50 values for *D. carinata* are significantly different at $p \leq 0.05$. ND – Could not be determined**

7.4.2.3. Group 3 experiments

The toxicities of equitoxic mixtures of each pesticide are presented in Figure 7.5. The toxicity of the equitoxic mixture of atrazine/molinate (Figure 7.5A) conformed to antagonism in Treatments 1 and 2 and to additivity in Treatments 3 and 4. In Treatment 5, the relationship conformed to synergism. The toxicity of atrazine/chlorpyrifos (Figure 7.5B) conformed to antagonism at TU values below 1.5 TU (Treatments 1 – 4) and then to additivity at higher TU values (Treatments 5 and 6). The toxicity of molinate/chlorpyrifos (Figure 7.5C) conformed to additivity. The toxicity of the three pesticide mixture (Figure 7.5D) conformed to antagonism (Treatment 1) and additivity (Treatments 2 and 3) and then to synergism in Treatments 4 and 5.

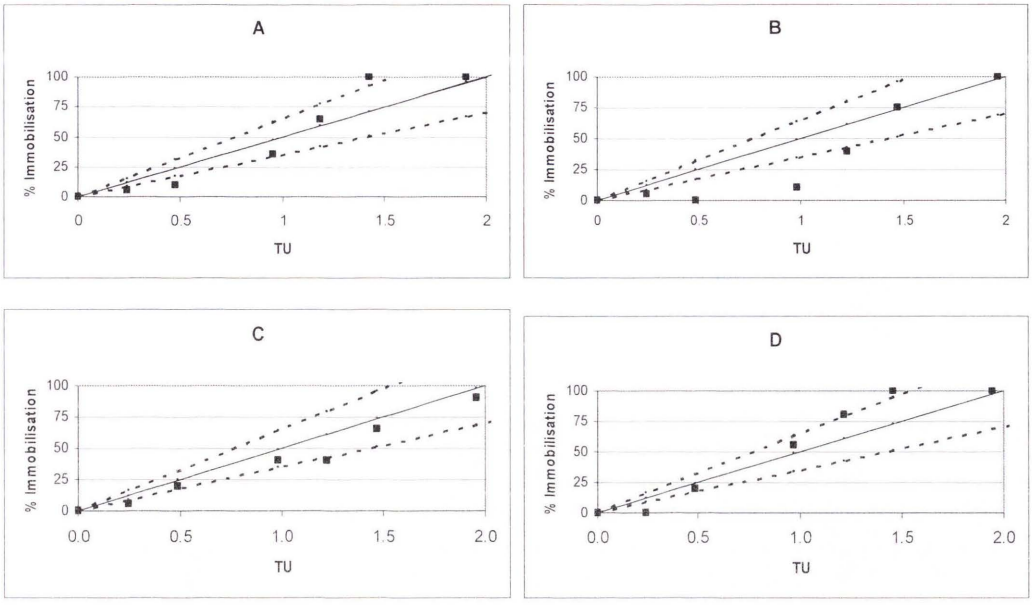

Figure 7.4: The toxicity of equitoxic mixtures of pesticides to *D. carinata* cultured in water with salinity of 6300μS/cm. A - Atrazine and molinate; B - Atrazine and chlorpyrifos; C - Molinate and chlorpyrifos, D - Atrazine, molinate and chlorpyrifos. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

There were no significant ($p > 0.05$) differences in the EC50 values of *D. carinata* cultured at 200 and 6300 μS/cm for each of the four pesticide mixtures (Table 7.8).

Table 7.8: The sum of the toxic units (TU) of mixtures of individual pesticides that caused 50% immobilisation of *Daphnia carinata*.

Mixtures	Cultured at 200 μS/cm	Cultured at 6300 μS/cm
atrazine and molinate	1.04 (0.91 – 1.19)	0.94 (0.80 – 1.10)
atrazine and chlorpyrifos	1.19 (1.11 – 1.27)	1.26 (1.15 – 1.39)
molinate and chlorpyrifos	1.15 (1.01 – 1.31)	1.26 (0.98 – 1.61)
atrazine, molinate, chlorpyrifos	0.92 (0.83 – 1.03)	0.77 (0.65 – 0.90)

7.4.2.4. Group 4 experiments.

The toxicity of mixtures of pesticides and salinity are presented in Figure 7.6. The toxicity of the atrazine/molinate/salinity mixture conformed to additivity (Treatments

1 and 2) and antagonism (Treatments 3 and 4) and back to additivity (Treatments 5 and 6) (Figure 7.6A). The toxicity of the atrazine/chlorpyrifos/salinity mixture (Figure 7.6B) and the molinate/chlorpyrifos/salinity mixture (Figure. 7.6C) almost entirely conformed to additivity. The toxicity of the atrazine/molinate/chlorpyrifos/salinity mixture (Figure 7.6D) conformed to synergism in Treatment 1 and to additivity in Treatment 2 and to synergism in Treatment 3. None of the complex pesticide and salinity mixtures that could be compared had significantly ($p > 0.05$) different EC50 values to *D. carinata* cultured at 200 and 6300 µS/cm (Table 7.9).

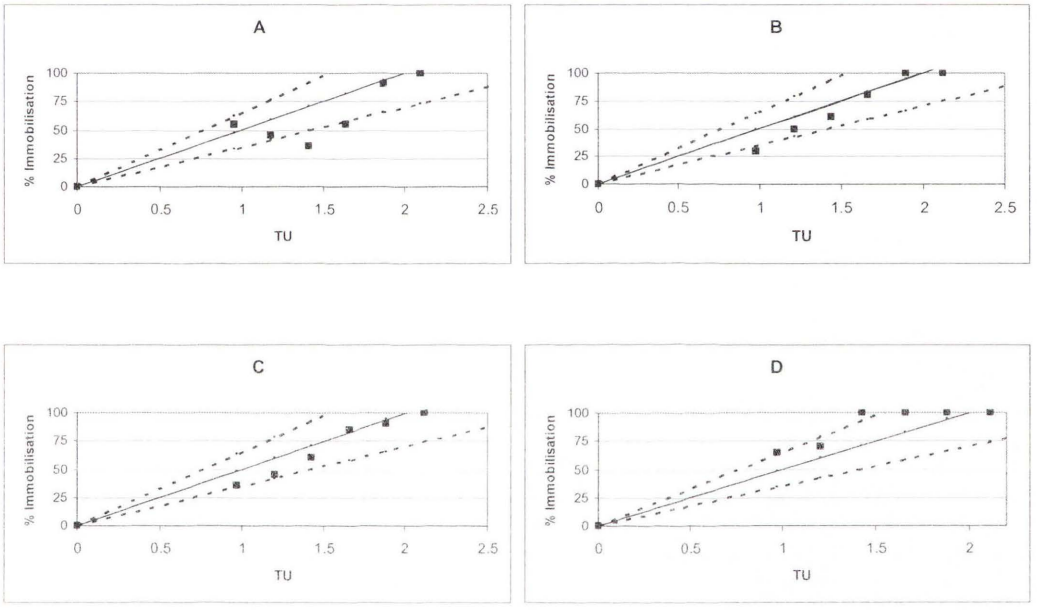

Figure 7.5: The toxicity of mixtures of pesticides with varying concentrations of salinities to *D. carinata* cultured in water with salinity of 6300µS/cm (the toxic contribution from the combination of pesticides equal to 1TU). A - Atrazine/molinate/salinity; B - Atrazine/chlorpyrifos/salinity; C - Molinate/chlorpyrifos/salinity ; D - Atrazine/molinate/chlorpyrifos/salinity. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic.

Table 7.9: The sum of toxic units (TU) of mixtures of pesticides and salinity that caused 50% immobilisation of *D. carinata*.

Toxic Combinations	EC50 TU cultured at 200 µS/cm	EC50 TU cultured at 6300 µS/cm
atrazine, molinate and salinity	1.06 (0.76 – 1.49)	1.51 (1.19 – 1.93)
atrazine, chlorpyrifos and salinity	1.16 (0.78 – 1.72)	1.24 (1.07 – 1.43)
molinate, chlorpyrifos and salinity	1.41 (1.21 – 1.64)	1.25 (1.04 – 1.50)
atrazine, molinate, chlorpyrifos and salinity	ND	ND

ND – Could not be determined

The types of toxicity relationships and the frequency of occurrence of each toxicity relationship of the different mixture combinations are presented in Table 7.10.

Table 7.10: The types of toxicity relationships observed in the mixture combination and frequency of their occurrence.

Toxic relationship	Frequency of occurrence
Additive	2
Changed from antagonism to additivity*	3
Changed from additivity to antagonism and back to additivity	2
Changed from antagonism to additivity then to antagonism and back to additivity*	3
Changed from antagonism to additivity and then to synergism*	3
Changed from synergism to additivity and back to synergism*	1
Total	14

*** - Changes in toxicity relationships occurred with increasing concentrations of mixtures.**

Of the fourteen toxicity mixtures two were additive, three combinations changed from antagonism to additivity, two changed from additivity to antagonism and then back to additivity, three changed from antagonism to additivity and again to antagonism then back to additivity, three changed from antagonism to additivity and then to synergism, and one changed from synergism to additivity and back to synergism.

In the high salinity *D. carinata* cultures there were 65 different combinations (64 in normal salinity cultures) of mixtures in the total of 14 mixtures studied. Table 7.11 summarises the number of the types and percent occurrence of toxicity relationships in these combinations.

Table 7.11: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos for *D. carinata* cultured at normal salinity and high salinity.

Toxicity relationship	*D. carinata* cultured at normal salinity (200 µS/cm)		*D. carinata* cultured at high salinity (6300 µS/cm)	
	Number of occurrence	% occurrence	Number of occurrence	% occurrence
Antagonistic	27	42.2	24	36.9
Additive	30	46.9	34	52.3
Synergistic	7	10.9	7	10.8

Approximately 37% of these mixtures conformed to antagonism, 52% to additivity and 11% conformed to synergism. The antagonistic relationships were reduced by about 5% in high salinity cultures compared with normal salinity cultures and additivity increased by the same percentage in high salinity cultures.

7.5. Discussion

The sensitivity of the cladocerans throughout the experiments remained stable, as shown in the cumulative summary chart of the reference toxicant tests (Figure 7.2). As the EC50 values of each test were within two standard deviations of the long-term mean EC50 value, it is considered that the relative sensitivities of the test organisms were stable over time and that there was no change in the sensitivity of *D. carinata* over the study period. This permits comparison of toxicity test results conducted at different times.

From the reference toxicant tests (i.e., salinity) conducted as part of the present study which provided acute EC50 values of salinity and from the work presented in Chapter 6, it appears that there is a threshold salinity required to induce adaptation to salinity. A salinity of 6300 µS/cm was deemed to be close to the salinity threshold with 40% of cases having higher EC50 values and hence signs of adaptation.

The sensitivity of the cladocerans cultured at 6300 µS/cm to the three pesticides tested individually was not significantly ($p > 0.05$) different compared to cladocerans

cultured at 200 μS/cm. Except for the 0.5 times the EC50 value of atrazine and salinity mixture, all the other mixtures tested did not show significant ($p > 0.05$) differences in their EC50 values between cladocerans cultured in 200 and 6300 μS/cm water. Thus, cladocerans cultured at 6300 μS/cm were able to maintain their physiological functions, including osmoregulation, without losing their ability to deal with other toxicants.

Rose et al. (2004) found that the development of tolerance by *Ceriodaphnia* cf. *dubia* to 3, 4-dichloroaniline was inversely related to the concentration of this chemical and the number of generations the cladoceran had been exposed and that tolerance could be induced within one generation. They argued that the different concentrations of 3, 4-dichloroaniline exerted different selective pressures, with cladocerans exposed to greater selective pressure (i.e., at higher concentrations) showing increased tolerance after fewer generations than those exposed to lower selective pressure. The 6300 μS/cm salinity treatment that *D. carinata* were cultured in was 71% of the salinity EC50 (immob) value for cladocerans cultured at 200 μS/cm. It would be expected that cladocerans cultured at high salinity (6300 μS/cm) for over a year (i.e., at least 13 generations) would have exerted a sufficiently high selective pressure on *D. carinata* to induce some change in acute tolerance and in the present study 40% of toxicity tests that used the high salinity acclimatised cultures were less sensitive to salinity (i.e., they had developed tolerance).

Present results show that cladocerans acclimatised to salinity over several generations, developed a certain degree of resistance to acute salinity toxicity, but there were no changes in response to the other toxicants studied. This could be due to the nature of salinity toxicity, which is sea water in this case. The actual effect of salinity seems to be due to physiological stress rather than toxicity of the ionic constituents of sea water. If a saline effluent, such as mine effluent, which has different ionic compositions (dominated by the more toxic divalent ions) (Yim et al. 2006), were used, the result could be different. The defence mechanism against trace metals is governed by metallothionin-like proteins (MTLP) (Guan and Wang 2006; Tsui and Wang 2007). So if the ionic toxicity has a dominant role in the toxic effect of salinity

and MTLP were involved a different toxic response may occur. Since our focus was on the effects of inland salinity in Australia, different compositions of salinity were not considered.

The "metabolic cost" hypothesis (Calow and Sibly 1990) states that toxic stress induces metabolic changes in organisms, which in turns leads to increased energy consumption resulting ultimately in adverse effects on growth and reproduction . It is well established that chronic estimates of toxicity are more sensitive (i.e., toxicity occurs at lower concentrations) than acute estimates of toxicity. Thus, a lower salinity threshold to induce changes in tolerance and subsequently measurable tolerance changes may have been detected at the salinities tested if chronic toxicity had been measured in the current study. This, in fact, was observed in Chapter 5 where several life history traits of *D. carinata* were adversely affected at salinities of 5500 μS/cm.

Implications of synergism in mixture toxicities at low concentrations

Before discussing the results of the mixture toxicity tests, it is necessary to explain a feature of some of the results – the tendency at high TU values for toxicity of the mixtures to change from conforming to synergism to conforming to additivity. This is a result of the nature of the experimental design of the toxicity tests rather than an actual change in the type of toxicity. This can best be explained by referring to a plot of a mixture toxicity test (Figure 7.6) – in this case the plot of the % immobilisation of *D. carinata* against the total TU in a mixture of atrazine/molinate/chlorpyrifos and salinity (see Figure 7.5D).

In Treatments 1 (which is the mixture of three pesticideS only) and 2, toxicity of the mixtures were consistent with additivity; however, with increasing salinity Treatment 3 changed to being consistent with synergism with 100% of *D. carinata* being immobilised. For the rest of the treatments (4, 5 and 6), 100% of the organisms were immobilised. Any further increases in the salinity could possibly immobilise more cladocerans but in practice this is not possible, as the number of cladocerans per treatment is controlled. However, it is not possible to know whether the toxicity has reverted to conforming to additivity or whether it is a function of the design of the

toxicity test. In this example, where there are a series of concentrations of the mixture with 100% immobilisation, the second explanation for the apparent conformity with additivity is the most likely explanation. This situation only applies if lower concentrations of the mixture are synergistic – it does not apply if the toxicity conforms to antagonism. Thus, correct interpretation of the results of mixtures is only possible up to the point where 100% immobilisation is first achieved.

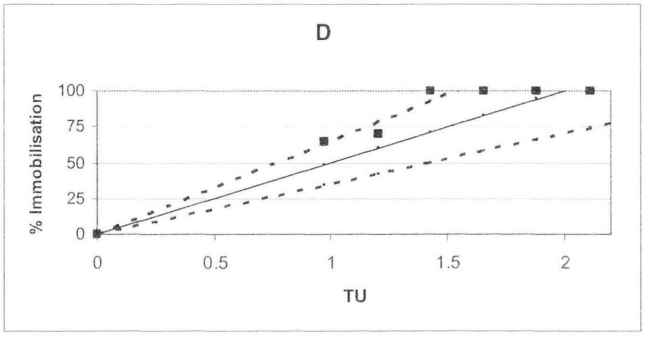

Figure 7.6: The variation in the toxicity of a mixture of atrazine, chlorpyrifos, molinate and salinity with increasing salinity (extracted from Figure 7.5). The TU values are for the entire mixture. Solid line represents the expected additivity. Two dashed lines indicate the 30% deviation from additivity. Mixtures that lie to the right of the lower dashed line are classed as antagonistic, while those that lie to the left of the upper dashed line are classed as synergistic. 100% immobilisation occurred in the 3rd treatment.

Mixture toxicity theory suggests that only mixtures containing chemicals with the same mechanism of action can have concentration additive (CA) toxicity (Warne 2003; Warne and Hawker 1995) However, the three test chemicals used in the present study have different mechanisms of action (i.e., atrazine inhibits photosynthesis by interfering with electron transport (Hill reaction), (Ebert and Dumford 1976), molinate inhibits cell division in plants (Sabater and Carrasco 1998) and chlorpyrifos inhibits acetylcholinesterase in animals . It is expected that mixtures of these chemicals would not conform to concentration additivity. However, about 52% of the combinations studied were additive while about 37% were antagonistic (Table 7.11). The combinations which showed synergism were about 11% and should be given due consideration as they exert more toxicity than expected in CA model. There was approximately a 5% decrease in antagonism in the high salinity culture compared with

the normal salinity culture and additivity increased by the same percentage in the high salinity culture while synergism remained the same in both cultures (Table 7.11).

Acclimation by an organism to a toxicant may produce different responses by the organism when exposed to other toxicants. Resistance usually results from genetically controlled biochemical or physiological changes from the normal type (Tsui et al. 2005). Sensitivity to toxic organic chemicals can be changed due to effects on their defence mechanisms such as cytochrome P450 which can metabolise the toxicants thereby reducing or increasing toxicity. Atrazine can increase the toxicity of organophosphorus insecticides such as chlorpyrifos by activating cytochrome P450, which metabolises chlorpyrifos to more toxic metabolites e.g., chlorpyrifos-*O*-analog (Belden and Lydy 2000; Pape-Lindstrom and Lydy 1997). However, in the current study, mixtures containing only atrazine and chlorpyrifos did not conform to synergism, rather they conformed to antagonism and/or additivity. Conformity to synergism only occurred when the mixture contained atrazine, molinate and chlorpyrifos.

The funnel hypothesis for mixtures (Warne and Hawker 1995) hypothesised and subsequently experimentally validated that as the number of components in equitoxic mixtures increases the likelihood of the toxicity of a mixture conforming to additivity increases. The equitoxic mixtures of atrazine/chlorpyrifos/molinate and of atrazine/chlorpyrifos/molinate/salinity that conformed to synergism contained more compounds than the atrazine/chlorpyrifos and the atrazine/chlorpyrifos/salinity mixtures that conformed to additivity. This indicates that molinate played a role in changing the toxicity of the mixtures from conforming to additivity to conforming to synergism. The mechanism by which molinate increased the toxicity of the mixture is yet unknown.

Given the ongoing salinisation of Australian inland waters and the concurrent exposure of organisms within those waterways to pesticides and mixtures of pesticides these findings are very positive. They clearly show that multigenerational exposure of *D. carinata* to elevated salinity (6300 µS/cm) did not affect their acute sensitivity to salinity, atrazine, chlorpyrifos, molinate, mixtures of these pesticides,

and mixtures of these pesticides with elevated salinity. Thus, at least for the cladoceran species and pesticides tested, there is little need for concern of increasing salinity exacerbating the ecological impacts of pesticides in Australian inland waterways. However, the effects of long-term exposure to elevated salinity on other species, toxicants and mixtures are not known, and therefore warrant investigation.

7.6. Conclusions

Acclimatisation of *D. carinata* over 13 generations to 6300 μS/cm induced a certain degree of tolerance to acute salinity toxicity. With one exception, acclimatisation to 6300 μS/cm water did not change the tolerance of *D. carinata* to acute exposures of atrazine, chlorpyrifos or molinate acting individually, as mixtures of the pesticides or as mixtures of the pesticides with elevated salinity. There are, thus, no adverse effects from the salinity acclimatisation of *D. carinata* on their defence mechanisms against the pesticides atrazine, molinate and chlorpyrifos or their mixtures.

Chapter 8

General Discussion

8.1. Introduction

The present study aimed at studying different exposure scenarios of the freshwater alga, *Pseudokirchneriella subcapitata* and the freshwater cladoceran, *Daphnia carinata* to salinity, atrazine, molinate and chlorpyrifos and mixtures of the above. The results are presented and discussed in Chapters 2 to 7. First, the effects of salinity on several generations of *P. subcapitata* and *D. carinata* were studied. Then a series of experiments with these test organisms cultured at their normal salinities (100 and 200 µS/cm for *P. subcapitata* and *D. carinata*, respectively) and elevated salinities (6000 and 6300 µS/cm for *P. subcapitata* and *D. carinata*, respectively) were conducted. Individual toxicities of salinity, atrazine, molinate and chlorpyrifos to *P. subcapitata* (chronic) and *D. carinata* (acute) were studied. Then different combinations of mixtures of salinity and three pesticides were tested with *P. subcapitata* and *D. carinata*. This approach was followed based on the fact that 1) exposure of Australian freshwater organisms to salinity has been a long term process (Jolly et al. 2001; Williams 1987; Williams 1999); 2) pesticides in the environment are usually present as mixtures (Faust, Altenberger et al. 1994; Van den Brink, Van Donk et al. 1995; Kungolos, Samaras et al. 1999; Faust, Altenburger et al. 2001); and 3) pesticide pollution is present in the same environment where salinity problems occur especially in irrigated agricultural areas.

8.2. Summary of the findings of each component of the study with regards to individual test species *P. subcapitata* and *D. carinata*

A summary of key findings of the experimental chapters for the two test species individually is discussed in this section.

Algae as primary producers play an important role in trophic relationships in limnetic systems as they are the primary contributor of energy to higher trophic levels. However, their contribution can only be maintained if environmental conditions are optimum. Salinity is one of many stressors that can impact on their functioning. Whether they can adapt to increasingly saline waters over several generations is little known. The present study on *P. subcapitata* exposed to increased salinity over several generations showed significant ($p \leq 0.05$) effects on their growth rates, cell division rates, cell yield and generation times at salinities of $\geq$ 3000 µS/cm. In addition, it increased its sensitivity to salinity over the generations in terms of growth rates, cell division rates, cell yield and generation times. *P. subcapitata* did not develop tolerance to the increasing levels of salinity when exposed over several generations. The data suggest that primary producers such as *P. subcapitata* do not readily adapt to increasing salinity and their loss thus may affect the next trophic level such as cladocerans in the environment.

The present studies on *D. carinata* exposed to a salinity of $\geq$ 5500 µS/cm showed significant ($p \leq 0.05$) reductions in reproduction, adult body lengths, intrinsic rate of natural increase and ingestion rates. Neonatal lengths and filtration rates were more sensitive to salinity being significantly affected at 2000 and 4000 µS/cm, respectively. Significant ($p \leq 0.05$) delays in the production of neonates also occurred with increasing salinities.Sensitivity also increased over generations for mean total reproduction and mean intrinsic rate of natural increase. *D. carinata* did not develop tolerance to increased levels of salinity over the generations. The long-term impacts of salinity on cladoceran populations may have consequences on zooplanktivorous species in the aquatic food chain.

Individual chronic toxicities (72-hour IC50 values based on growth inhibition) of salinity, atrazine, molinate and chlorpyrifos to *P. subcapitata* were approximately 5600 µS/cm, 48 µg/L, 300 µg/L, 800 µg/L, respectively. Based on the toxicant classification (US EPA 2006), atrazine was very highly toxic, molinate was highly toxic and chlorpyrifos was moderately toxic (just below "highly toxic") to the alga. About 50% of toxicity mixture combinations conformed to antagonism, 47% to additivity and 2% to synergism. Since the proportion of synergistic effects was low, water quality guidelines (WQGs) based on the concentration addition (CA) model should provide sufficient protection for the species concerned assuming the mixtures tested are representative of all mixtures.

Acute toxicities of pesticides to *D. carinata* (EC50 immobilisation) in descending order were chlorpyrifos (0.213 µg/L), molinate (25.1 mg/L) and atrazine (42.4 mg/L). Chlorpyrifos was very highly toxic while atrazine and molinate were both slightly toxic to *D. carinata*. Salinity caused 50% immobilisation of *D. carinata* at 8813 µS/cm. The toxicities of mixtures of pesticides and salinity results showed 47% to conform to additivity, 11% to synergism and 42% to antagonism. The relatively high proportion of mixtures conforming to synergism needs attention in refining WQGs, although the occurrence of synergism was similar to that in other mixture studies.

Exposure of *P. subcapitata* to high salinity (i.e., 6000 µS/cm) over five successive 72 hour long cultures did not affect the sensitivity to the pesticides atrazine, chlorpyrifos, molinate and salinity individually. About 70% of mixtures did not show sensitivity changes (in terms of IC50 values). In mixtures of the pesticides and salinity, the majority of the concentrations (71%) tested conformed to additivity. Approximately 20% and 9% of the mixtures conformed to antagonism and synergism, respectively. There were changes in toxicity relationships in mixtures between non-acclimatised and acclimatised cultures i.e., antagonistic mixtures in non-acclimatised cultures became additive in acclimatised cultures. There were changes in sensitivity to the toxicant mixtures in the acclimated algae at a salinity of 6000 µS/cm.

Acclimatisation of *D. carinata* over 13 generations to 6300 µS/cm induced a certain degree of tolerance to acute salinity toxicity. With one exception (the mixture of

atrazine 0.5 TU x EC50 and salinity), acclimatisation to 6300 µS/cm water did not change the tolerance of *D. carinata* to acute exposures of atrazine, chlorpyrifos or molinate individually, as mixtures of the pesticides and as mixtures of the pesticides with elevated salinity. No adverse effects from salinity acclimatisation of *D. carinata* on their defence mechanisms against the pesticides atrazine, molinate and chlorpyrifos or their mixtures were found. Approximately 37% of the tested mixtures conformed to antagonism, 52% to additivity and 11% to synergism. The latter group may cause more toxicity than expected by the CA model. There was about a 5% decrease in antagonism in high salinity cultures compared with normal salinity cultures and additivity increased by the same percentage in high salinity cultures while synergism remained same in both cultures.

8.3. Relative sensitivity of *P. subcapitata* and *D. carinata* to salinity at multigenerational level and at chronic level (for *P. subcapitata)* and acute level (for *D. carinata*).

In this section, effects of multigenerational exposure to salinity as well as short-term exposure of salinity on non-acclimatised and acclimatised test species will be discussed.

8.3.1 Population level effects and life-history traits of long-term exposure to elevated salinity on *P. subcapitata* and *D. carinata.*

In the natural environment, exposure to certain toxicants i.e., salinity can occur throughout the life span of the organism and over generations. For this reason long-term chronic toxicity experiments are more environmentally realistic and preferred over short-term experiments. However, both acute and chronic toxicity tests generally only measure individual level traits and do not provide information about likely effects at higher levels of biological organization such as population and community levels. This is a major limitation of such tests, as ecotoxicology is or should be primarily concerned with effects at the population or at higher levels of biological

organisation (Bechmann 1994; Forbes and Calow 1999; Walthall and Stark 1997). Life-history tests and multigenerational studies (Hammers-Wirtz and Ratte 2000; Rose et al. 2000; Rose et al. 2002b) provide much more comprehensive information on how the toxicants affect populations and whether the sensitivity of organisms changes over time. Despite the distinct advantages associated with conducting these more environmentally relevant toxicity tests, very few are conducted principally because they are considerably more time consuming, labour intensive and hence costlier than acute and chronic tests.

Salinisation of ground and surface water is a gradual process occurring over timescales of decades rather than days or months (Jolly et al. 2001; Williams 1987; Williams 1999). Therefore the two test species, *P. subcapitata* and *D. carinata*, representing primary producers and primary consumers, were separately subjected to multigenerational salinity studies, which provided the required information on the effects of long-term salinity exposure (i.e., salinity acclimation) on life history characteristics. The two species were tested, after acclimation, for sensitivity to salinity as a result of salinity acclimation, using acute (for *D. carinata*) and chronic (for *P. subcapitata*) endpoints.

Table 8.1 summarises the common multigenerational endpoints studied for both *P. subcapitata* and *D. carinata*.

Table 8.1: The lowest observed effect concentration (LOEC) values for salinity to each generation of the alga *Pseudokirchneriella subcapitata* and the cladoceran *Daphnia carinata* for the endpoints that were common to both sets of multigenerational tests.

Toxic endpoint	The lowest observed effect concentrations (LOEC values) (µS/cm) for each generation			
	F0	F1	F2	F3
Growth Rate				
P. subcapitata	3000	> 6000	3000	3000
D. carinata	> 6300	5500	5500	5500
Generation Time				
P. subcapitata	3000	> 6000	6000	3000
D. carinata	> 6300	4000	6300	5500

In relation to these endpoints, *P. subcapitata* is more sensitive to the long-term effects of salinity with LOEC values mainly occurring at 3000 µS/cm than *D. carinata* for which all the LOEC values for were greater than or equal to 5500 µS/cm, with one exception of a LOEC value of 4000 µS/cm (Table 8.1).

In addition, the multigenerational LOEC values for salinity to *D. carinata* for reproduction, feeding rate and neonatal and adult body length were consistently greater than or equal to 5500 µS/cm. The multigenerational LOEC values for salinity to *P. subcapitata* cell division per day and cell yield were consistently greater than or equal to 3000 µS/cm. Thus, the salinity LOEC values for the various endpoints measured for each species were very consistent.

The multigenerational effects of salinity at 3000 µS/cm are all population level effects and therefore the effects will lead to lower densities of *P. subcapitata*. If it is assumed that other algal species have a unimodal distribution of sensitivities to salinity around that of *P. subcapitata* (not an unrealistic assumption) then at salinities of 3000 µS/cm or greater the availability of food for cladocerans will be significantly reduced and further increases in salinity will further decrease the availability of food. The selection of food by cladoceran is governed by the size of the food particles. The larger bodied cladocerans i.e., *D. magna* and *D. carinata,* which have coarse filters retain relatively larger particles i.e. algae (Brendelberger 1991). Smaller bodied cladocerans retain relatively smaller food particles i.e., bacteria (Brendelberger 1991; Geller and Muller 1981). It was described that phytoplankton as being more important as a food in providing nutrients for growth of *Daphnia* sp (Muller-Solger et al. 2002). Therefore reductions or limitations in the amount of algae available for cladocerans will affect their viability. Previous research has shown a series of effects by food of toxicological relevance to cladocerans. The effects can be categorised under maternal nutrition, and postnatal nutrition. Maternal nutrition can affect brood size and hence the body size of neonates (Glazier 1992; Gliwicz and Guisande 1992). Small neonates tend to be more sensitive to starvation (Gliwicz and Guisande 1992), and to some toxicants (Enserink et al. 1990). Therefore, the nutritional status of laboratory cultures may have a substantial effect on the sensitivity of neonates used in toxicity testing. The algal food concentration found in the natural environment could be even lower than that under

experimental conditions. Exposure to low food levels during chronic toxicity tests may affect the sensitivity of cladocerans to certain toxicants, including 3,4-dichloroaniline (Kluttgen et al. 1996), copper (Winner et al. 1977), cadmium (Chandini 1989) and lead (Enserink et al. 1995). In addition, maternal nutrition may affect the acute sensitivity of their offspring to some toxicants (Enserink et al. 1990). The influence of postnatal food conditions also becomes more important over time (Enserink et al. 1995). The effect of low food concentration on the chronic toxicity of a range of organic chemicals, i.e., sodium dodecyl sulphate (Martinez-Jeronimo and Garcia-Gonzalez 1994), diethylenetriamine pentaacetic acid (Van Dam et al. 1995), esfenvalerate (Barry et al. 1995), endosulfan (Barry 1996; Barry et al. 1995), fenoxycarb and chlorpyrifos (Rose et al. 2002a) and 3,4-dichloroaniline (Barry, Logan et al. 1995; Barry 1996; Kluttgen, Kuntz et al. 1996; Rose, J. et al. 2002) leads to increased, decreased and no change in sensitivity. Therefore, the nature of the effect appears to depend upon both the toxicant and endpoint in question.

Significant reduction in food availability can cause a variety of effects on the life history and sensitivity of cladocerans as described above. The effects on alga at comparatively low salinity levels (3000 µS/cm) could cause indirect effects on cladocerans, limiting the food availability, apart from the direct multigenerational effects on cladocerans visible at salinities ≥5500. The direct effects as well as the indirect effects have cascading effects on the species in subsequent trophic levels in the food chain. Therefore the direct toxicity of salinity and the decline of food sources (due to the effects of salinity on other species in the food chain) would affect the whole community inhabiting the same environment, since they are linked through trophic relationships.

8.3.2. Relative sensitivity of salinity acclimatised and non-acclimatised D. carinata to acute exposure and *P. subcapitata* to chronic exposure to salinity

The acute toxicity of *D. carinata* acclimatised and non-acclimatised to salinity and the corresponding chronic toxicity of *P. subcapitata* to salinity have been discussed separately in previous chapters of the thesis. The salt-acclimatised *P. subcapitata* did not acquire tolerance to toxicity of salinity, while the salt-acclimatised *D. carinata* did

develop tolerance and became significantly ($p \leq 0.05$) less sensitive to increased salinity.

When the relative sensitivity of *P. subcapitata* and *D. carinata* to salinity were compared, both the salt-acclimatised and non-acclimatised *P. subcapitata* were significantly ($p \leq 0.05$) more sensitive to salinity than the corresponding cultures of *D. carinata* (Figure 8.1) although there were no significant differences between acclimatised and non-acclimatised cultures of the same species. It is not usual to determine the relative sensitivity of organisms by comparing acute toxicity values for one species with chronic toxicity values for another species. However, in this case this is appropriate as we are comparing the effects of elevated salinity pulses of relatively short-duration (48 - 72 hours). It just happens that such durations correspond to acute exposure for the relatively long-lived cladoceran but to chronic exposure for the short-lived alga.

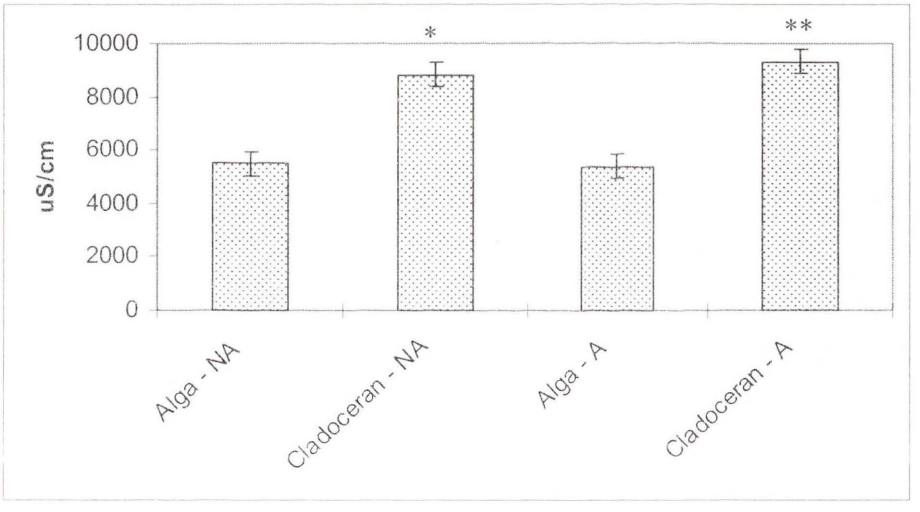

Figure 8.1: Mean IC50 (72 hour growth inhibition) and EC50 (48 hour immobilisation) of salinity for *P. subcapitata* and *D. carinata* acclimatised and non-acclimatised to salinity. Error bars = standard errors of means. NA = Non-acclimatised. A= Acclimatised. * = Non-acclimatised *D. carinata* is significantly different ($p \leq 0.05$) from *P. subcapitata* non-acclimatised. ** = Acclimatised *D. carinata* is significantly different ($p \leq 0.05$) from *P. subcapitata* acclimatised.

Given the above results and that algae such as *P. subcapitata* are the main food source for cladocerans such as *D. carinata* it is likely that cladocerans will experience indirect effects of toxicity from short term pulses of salinity at concentrations below

those that would cause direct toxic effects. As stated earlier the lack of food could have a range of effects including reduced reproduction and population growth rates and changes in sensitivity to other toxicants. When this finding is combined with the similar finding from the multigenerational studies it provides a strong argument that cladocerans may experience indirect effects due to exposure to elevated salinity of any duration at salinities below those that cause direct effects.

In addition, limited food (due to salinity effects on algal populations) could affect the physiological status of cladocerans thereby compromising their tolerance to toxicants. Therefore, it is possible that the actual effects in the environment at the salinities that should cause direct toxic effects may be greater than expected. This situation could be highly significant in a country like Australia where salinisation has occurred over a long period (with further increases predicted) and potential exposure may continue into the future.

8.3.3. Developing Acute to Chronic Ratios (ACRs)

Acute to chronic ratios (ACRs) are widely used in ecotoxicology to provide an estimate of the chronic toxicity of a chemical to a particular species when only acute data exists. They are used for this purpose in the Australian and New Zealand (ANZECC and ARMCANZ 2000), Canadian (CCREM 1991), South African (Roux et al. 1996) and USEPA (USEPA 1994b) water quality guidelines. This is done by dividing the acute toxicity value for a chemical to a particular species by the ACR or the geometric mean of ACRs for that species for the same chemical to another species. The use of ACRs has been criticized for a number of reasons. First, using an ACR implies that the mechanism of action of a chemical is the same under acute and chronic exposures. Evidence regarding this is conflicting (Baird et al. 1990; Mayer and Ellersieck 1986; Mayer et al. 1994). Second, the ratio obtained for one species may not be valid for the species that it is applied to (Calabrese and Baldwin 1993). Third, the magnitude of the ACR can vary markedly when comparing different biological endpoints when deriving the ACR and also the degree of desired protection may vary.. Despite these limitations, the use of ACR is likely to continue due to the fact that the vast majority of toxicity data generated is from acute tests.

Acute to chronic ratio values for *D. carinata* to salinity were calculated for both the acclimatised and non-acclimatised cultures according to the method described by Warne (2001) (Table 8.2). Equivalent ratios were calculated for the acclimatised and non-acclimatised *P. subcapitata*, however; in this case they are not strictly ACR values as they are based on the results of short-term chronic toxicity tests and chronic tests conducted as part of the multigenerational studies. The ACR values of non-acclimatised *P. subcapitata* were significantly ($p \leq 0.05$) larger than those of the acclimatised cultures. For cladocerans, the ACR values of non-acclimatised cultures were significantly smaller ($p \leq 0.05$) than those of acclimatised cultures. When the ACRs of the two species with the same acclimation history were compared (e.g. *D. carinata* non-acclimatised and *P. subcapitata* non-acclimatised) no significant ($p > 0.05$) differences were observed.

Table 8.2: The acute to chronic ratios (ACRs) of non-acclimatised and acclimatised *Pseudokirchneriella subcapitata*. (derived from short-term chronic IC50 and multigenerational lowest observed effect concentration (LOEC)) and *Daphnia carinata* (derived from acute EC50 and chronic/multigenerational LOEC).

Culture for alga /generation for cladoceran	Non-acclimatised to salinity		Acclimatised to salinity	
	P. subcapitata	*D. carinata*	*P. subcapitata*	*D. carinata*
0	0.94	1.6	0.87	1.7
1	1.87	1.4	1.73	1.48
2	1.87	1.6	1.73	1.7
3	1.87	1.6	1.73	1.7
4	1.87		1.73	

All the ACR values lie within the range of 0.8 to 2, which is relatively small. No ACR values for salinity (sea water) are available to compare with the results of present study. The ACR values for NaCl and for Na_2SO_4 for *Caridina nilotica*, a freshwater shrimp species were 2.6 and 3.17 respectively (Slaughter 2005). These ACR values were greater than the salinity ACR values of the present study. ACR values similar to the present study were reported for non-polar chemicals (Roex et al. 2000). Considerably larger ACRs are often reported for pesticides (Ferrando et al. 1996), heavy metals (Spehar and Carlson 1984) and a wide range of organic toxicants (Roex et al. 2000).

8.3.4. Incorporating potential indirect effects of salinity into guidelines for salinity in freshwater ecosystems.

Currently, salinity is not treated as a toxicant in the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ 2000). Rather, it is treated simply as a chemical property of aquatic ecosystems. As such the limit for salinity is set as the 80 percentile of salinity data for freshwater bodies (i.e., upland and lowland rivers, lakes) that are considered to be little modified. However, Kefford et al. (2006, 2005, 2002) argued that salinity should be treated as a toxicant and therefore WQGs should be derived using the species sensitivity distribution (SSD) method. The vast majority of toxicity data including that for salinity has been conducted by exposing individuals of a single species to a single toxicant in ambient environmental media (usually fresh water). Such toxicity tests have been validly criticised because: the routes of exposure are environmentally unrealistic and too simple (e.g. exposure to a constant concentration of the toxicant); the experiments do not take into account variation in environmental conditions that occurs in the field; they do not account for the variation in wild populations over time; they only examine species specific responses; they do not account for species interactions, the possibility of indirect effects or possible bioaccumulation effects (eg. Graney et al. 1995; Ward and Jacoby 1995; Warne et al. 1998).

Species sensitivity distribution methods use all of the available toxicity data to derive a curve showing the range of species sensitivities, then derive a guideline value that protects a given percentage, e.g., 95%. Therefore the limitations of the toxicity data become incorporated into the results of the SSD method, such as in developing water quality guidelines.

The current project has clearly shown that the alga *P. subcapitata* and the cladoceran *D. carinata* have markedly different sensitivities to salinity, with the alga being the more sensitive (Figure 8.1). Two potential reasons for this are that the cladoceran has an exoskeleton covering most of its surface area, and this would be less permeable to salt ions than an algal membrane. In addition, the physiology of the cladocerans is more evolved than that of alga and permitting active osmoregulation.

This study has also shown that there is the potential for cladocerans to experience indirect toxic effects of elevated salinity at salinities well below those that would cause direct toxic effects. Kefford et al. (2006) used acute toxicity data for salinity to macroinvertebrates from Goulburn, Broken, Campaspe and Loddon river catchments in southern Murray-Darling basin in Victoria, Australia and a SSD method to derive salinities that should protect various percentages of macroinvertebrates in freshwater ecosystems (Table 8.3). As they used acute toxicity data and water quality guidelines are designed to protect organisms and ecosystems from life-long exposure they used a range of safety factors to convert the acute data to estimates of chronic toxicity (Table 8.3).

Direct acute effects of short duration exposure to salinity on the cladoceran *D. carinata* occurred at salinities $\geq$ 8800 µS/cm but chronic effects to the alga *P. subcapitata* occurred at $\geq$ 5600 µS/cm. Therefore, the threshold for indirect toxic effects on *D. carinata* could be 5600 µS/cm. When both species had been exposed for multiple generations to elevated salinity, threshold toxic effects to *D. carinata* and *P. subcapitata* occurred at $\geq$ 5500 µS/cm and $\geq$ 3000 µS/cm, respectively. If it is assumed that all the macroinvertebrates used in the SSD by Kefford et al. (2006) relied predominantly on algae as their food source and that each macroinvertebrate and its food source had the same ratio between their sensitivities as *P. subcapitata* and *D. carinata* to salinity then all the protective concentration values would be 64% of the values derived by Kefford et al. (2006) (i.e., 5600/8800 x 100) for short term pulse exposures and the species sensitivity distribution would shift to the left. If however, exposure was multigenerational then all the protective concentration values would become 55% as large as the values derived by Kefford et al. (2006) (i.e., 3000/5500 x 100) and again the species sensitivity distribution would shift to the left. The effect that this would have on the results of the SSD analysis of the 110 macroinvertebrate data is shown in Table 8.3 using the correction for short-term pulse exposures (i.e., 64% of their original values). However whilst the above analysis is interesting it should be noted that the current water quality guidelines (WQGs) for salinity in Australia and New Zealand are not based on toxicity data but on the 80th percentile of ambient background concentrations in minimally disturbed ecosystems (ANZECC and ARMCANZ 2000).

When considering the above analysis the assumptions required to permit the calculations presented in Table 8.3 are large and over-simplistic, however the results do clearly indicate that current water quality guidelines may well be under-protecting aquatic ecosystems. A limitation of the Kefford et al. (2006) data set is that it only includes macroinvertebrates. Whereas the current Australian and New Zealand WQGs require toxicity data to at least five species that belong to four taxonomically different group in order to use a SSD approach. Having said that, the Kefford et al. (2006) data set is probably the single best data set available for the toxicity of salinity to Australian organisms.

Table 8.3: Calculated protective concentrations of salinity (mS/cm) for selected percentages of taxa with safety factors (extracted from Kefford et al. (2006) based on direct toxic effect data and having accounted for indirect effects from short-term exposures (revised protective concentration values are in parentheses).

% of taxa protected (SE)*	Safety factors				
	20	10	5	3.33	0 (no safety factor)
98.9 (1.1)	0.40 (0.26)	0.80 (0.51)	1.60 (1.02)	2.40 (1.54)	8.00 (5.12)
95.6 (2.1)	0.58 (0.37)	1.16 (0.74)	2.32 (1.48)	3.48 (2.23)	11.60 (7.42)
94.6 (2.4)	0.63 (0.40)	1.26 (0.81)	2.52 (1.61)	3.78 (2.42)	12.60 (8.06)
92.4 (2.8)	0.64 (0.41)	1.28 (0.82)	2.56 (1.64)	3.84 (2.46)	12.80 (8.19)
89.9 (3.2)	0.69 (0.44)	1.38 (0.88)	2.76 (1.77)	4.14 (2.65)	13.80 (8.83)
82.1 (4.2)	0.91 (0.58)	1.82 (1.16)	3.64 (2.33)	5.46 (3.49)	18.20 (11.65)
79.4 (4.5)	0.94 (0.60)	1.88 (1.20)	3.76 (2.41)	5.64 (3.61)	18.80 (12.03)
50.9 (6.3)	1.50 (0.96)	3.00 (1.92)	6.00 (3.84)	9.00 (5.76)	30.00 (19.20)
19.6 (6.7)	2.30 (1.47)	4.60 (2.94)	9.20 (5.89)	13.80 (8.83)	46.00 (29.44)

SE* – standard error

8.4. Toxicity of individual pesticides atrazine, molinate and chlorpyrifos on *P. subcapitata* (chronic) and *D. carinata* (acute) and effects of acclimation to salinity.

The off-site migration of pesticides used for crop protection can exert deleterious effects on non-targeted species in the aquatic environment. In Australia, the same aquatic ecosystems that are potentially exposed to pesticides are also subject to

salinisation. Therefore, the present study determined the toxic effects of the pesticides atrazine, molinate and chlorpyrifos acting individually and in mixtures including salinity on an alga (*P. subcapitata*) and a cladoceran (*D. carinata*).

8.4.1. Relative sensitivity of *P. subcapitata* and *D. carinata* to atrazine, molinate and chlorpyrifos.

The individual toxicities of the herbicides, atrazine and molinate, and the insecticide chlorpyrifos on the two test species were determined on populations non-acclimatised and acclimatised to elevated salinity. The EC50 and IC50 values and the corresponding 95% confidence limits for the pesticides are presented in Table 8.4.

Table 8.4: The toxicity (EC50 or IC50 and 95% confidence limits in parentheses) of atrazine, molinate and chlorpyrifos to acclimatised and non-acclimatised *P. subcapitata* and *D. carinata*.

Toxicant	Non-acclimatised EC50 or IC50 (µg/L)		Acclimatised EC50 or IC50 (µg/L)	
	P. subcapitata	*D. carinata*	*P. subcapitata*	*D. carinata*
Atrazine	47.8* (27.1 – 63.5)	4250* (3930 – 4590)	42.7* (32.4 – 53.9)	4150* (3710 – 4640)
Molinate	300* (262 – 316)	2540* 2320 – 2700)	282* (240 – 311)	2750* (2400 – 3150)
Chlorpyrifos	797* (673 – 1128)	0.21* (0.19 – 0.23)	1117* (900 – 1460)	0.18* (0.17 – 0.20)

* - means that *P. subcapitata* and *D. carinata* with the same acclimatisation history (i.e. acclimatised or non-acclimatised) were significantly different ($p \leq 0.05$).

The alga *P. subcapitata* is highly susceptible to the two herbicides atrazine and molinate. Atrazine inhibits electron transport in the Photo-system II, which causes destruction of the chloroplast (Torres and O'Flaherty 1976), inhibition of carbon uptake and carbohydrate synthesis (Larsen et al. 1986; Solomon et al. 1996). Since the alga is an autotrophic organism, effects on the chloroplast could cause serious effects on alga growth and survival. Molinate inhibits algal cell division (Tomlin 1994) and also causes physiological interference with the metabolism of proteins, as well as binding to the special proteins . Since there is no definitive pathway for chlorpyrifos

to cause toxicity in the alga, it is the least toxic of the three test chemicals to *P. subcapitata*.

In the case of *D. carinata* there are no identified pathways for herbicides to exert their toxic effects upon. However studies have shown that atrazine and molinate cause acute as well as chronic toxic effects to cladocerans (Julli and Krassoi 1995; Foster, Thomas et al. 1998; Phyu, Warne et al. 2004). Chlorpyrifos is highly toxic to cladocerans and the mode of action is the inhibition of the enzyme acetylcholinesterase, resulting in the accumulation of the neurotransmitter, acetylcholine, at nerve endings and the rapid use of all ATP (Giesy et al. 1999; Straus and Chambers 1995).

Data generated showed that the alga, *P. subcapitata* was more sensitive to some toxicants i.e. herbicides compared with the cladoceran, D. carinata. Therefore, D. carinata may experience indirect effects at lower concentrations than those indicated by toxicity tests using D. carinata alone. The insecticides i.e. chlorpyrifos are highly toxic to D. carinata due to mode of action.

Limited information on the toxicity of mixtures of the three pesticides is available. Atrazine enhances the toxicity of chlorpyrifos to invertebrates by the formation of chlorpyrifos oxon by oxidative desulfuration (Pape-Lindstrom and Lydy 1997; Giesy, Solomon et al. 1999; Belden and Lydy 2000; Eisler 2000). Even though no information is available on the mixture effects of molinate/atrazine and molinate/chlorpyrifos combinations, the above mentioned physiological and biochemical effects could certainly have combined and caused effects on the algae and cladocerans.

The sensitivity of both the acclimatised and non-acclimatised alga to the herbicides, atrazine and molinate, were approximately one hundred-fold higher than that of the cladoceran. Thus, in addition to direct toxicity of the two herbicides, the cladocerans would be experiencing indirect effects even at lower concentrations of herbicides, as a result of declining algal numbers available as food.

In contrast, for chlorpyrifos, the acclimatised and non-acclimatised cladocerans were approximately 6000 and 3500 times more sensitive than the correspondingly acclimatised alga. In this scenario algal populations are likely to increase as a result of the lack of grazing pressure from herbivorous zooplankton (Papst and M.G.Boyer 1980). These types of phenomena actually lead to disruption of the community structure. The reduction of grazing can cause conditions leading to algal blooms, which in turn have adverse impacts on the health of the system, creating low dissolved oxygen, reducing light penetration and resulting in fish kills (apart from direct toxicity of the pesticide), etc. (Scholten 2005).

8.5. Toxicities of different combinations of mixtures of salinity, atrazine, molinate and chlorpyrifos *P. subcapitata* and *D. carinata* non-acclimatised and acclimatised to salinity over generations.

As discussed previously, salinity is quite common in irrigated areas where there is heavy use of agricultural pesticides. Because of that, the combined effects of mixtures of pesticides and salinity need to be studied to understand the toxicological consequences of such mixtures on aquatic organisms. Different combinations of salinity and individual pesticides, equitoxic mixtures of pesticides and salinity and mixtures of pesticides, were examined in the present study. It is not possible to compare the IC50 or EC50 values for the toxicity mixtures since they are based on toxic units derived from 50% effects of individual toxicants and therefore are completely different in toxicant concentrations. Therefore, the percentage occurrence of toxicity relationships i.e. additivity, antagonism and synergism in different toxicity combinations were used to discuss the relative toxicities of different mixtures to the two species. Table 8.5 summarises these toxicity relationships in different mixture combinations of salinity and pesticides for non-acclimatised *P. subcapitata* and *D. carinata*.

Table 8.5: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos for non-acclimatised *P. subcapitata* and *D. carinata*.

Toxicity relationship	% occurrence for *P. subcapitata*	% occurrence for *D. carinata*
Antagonistic	50.9	42.2
Additive	47.3	46.9
Synergistic	1.8	10.9

Approximately 47% of mixtures conformed to additivity for both species, 51% of mixtures conformed to antagonism for *P. subcapitata* while about 42% mixtures conformed to antagonism for *D. carinata*. For *P. subcapitata* only about 2% of mixtures conformed to synergism whereas for *D. carinata*, 11% conformed to synergism. Therefore, *D. carinata* seemed to be more sensitive in terms of the toxicity relationships to the mixtures studied compared with *P. subcapitata*. For both species approximately 90% of the tested mixtures conformed to antagonism or additivity, which indicates that the use of the CA model with appropriate safety factors, to account for the synergism, will provide sufficient protection of primary producers and primary consumers in the system.

Table 8.6 summarises the toxicity relationships of different mixture combinations of salinity and pesticides for salinity acclimatised *P. subcapitata* and *D. carinata* and Table 8.7 presents the percentage difference of each toxicity relationship between salinity acclimatised and non-acclimatised test species.

Table 8.6: Types of toxicity relationships in different mixture combinations of salinity and pesticides atrazine, molinate and chlorpyrifos for acclimatised *P. subcapitata* and *D. carinata*.

Toxicity relationship	% occurrence for *P. subcapitata*	% occurrence for *D. carinata*
Antagonistic	20.0	36.9
Additive	70.9	52.3
Synergistic	9.1	10.8

Acclimation to salinity considerably impacted on the toxicity relationship of mixtures to *P. subcapitata* as indicated by the changes percentage of mixtures that conformed to antagonism, additivity and synergism (compare Tables 8.5 and 8.6). Antagonistic relationships of non-acclimatised algal populations were reduced by approximately 31% and additivity and synergism increased by approximately 24% and 7%

respectively. These observations showed that the alga became more sensitive to mixtures studied due to the multigenerational exposure to salinity. The changes in sensitivity of *P. subcapitata* observed in the multigenerational salinity experiments were further supported by the present results.

Table 8.7: Percentage difference of occurrence of different toxicity relationships among the salinity acclimatised and non-acclimatised *P. subcapitata* and *D. carinata* non-acclimatised and acclimatised.

Toxicity relationship	% occurrence for non-acclimatised	% occurrence for acclimatised	% difference
P. subcapitata			
Antagonistic	50.9	20	-30.9
Additive	47.3	70.9	+23.6
Synergistic	1.8	9.1	+7.3
D. carinata			
Antagonistic	42.2	36.9	-5.3
Additive	46.9	52.3	+5.4
Synergistic	10.9	10.8	-0.1

Percentage occurrence to toxicity relationships on non-acclimatised *D. carinata* and changes of these relationships as a result of salinity acclimation is different to that of *P. subcapitata*. A slight increase in sensitivity in acclimatised *D. carinata* was indicated by an approximately 5% decrease in antagonistic relationships and by an equivalent 5% increase in additive relationships. The percentage of mixtures that conformed to synergism was not affected by salinity acclimatisation. Thus, there were no substantial effects of salinity acclimation on the toxicity of mixtures to *D. carinata*. However, the increase in sensitivity of *P. subcapitata* will have indirect effects on cladocerans due to a possible reduction of algal food for cladocerans. It is difficult to work out such effects quantitatively in relation to mixtures. The mean percentage occurrence of each toxicity relationship for the two test species together with changes in salinity acclimation are presented in Table 8.8.

Table 8.8: Mean percentage of *P. subcapitata* and *D. carinata* non-acclimatised and acclimatised both species together

Toxicity relationship	% occurrence for non-acclimatised	% occurrence for acclimatised	% difference
Antagonistic	46.5	29.2	-17.3
Additive	47.1	60.8	+13.7
Synergistic	6.4	10.0	+3.6

Non-acclimatised cultures showed antagonism and additivity in the same proportions while salinity acclimation decreased antagonism by approximately 17% and increased additivity by approximately 14% and synergism by 3.6%. Water quality guidelines based on the CA model would provide sufficient protection for the species because for both salinity acclimatised and non-acclimatised cultures, the mean (of both test species) of mixtures that conformed to antagonism and additivity was 90% and 93% respectively. Deneer (2002), Faust et al. (1994), Warne and Hawker (1995) and Ross and Warne (1997) found that approximately 85 - 95% of mixtures (irrespective of the type of chemical) were additive and therefore can be predicted with reasonable accuracy by the CA model. The additivity percentage of the present study is however considerably smaller than those observations (i.e., approximately 47% and 61% for salinity acclimatised and non-acclimatised, respectively). As described by Warne (1992), when the number of component mixtures increases the toxicity relationship tends to conform to additivity. Since the maximum number of components in the present study was four, the deviation from additivity can be understood. The 10% of mixtures that conformed to synergism will not be adequately protected by using the CA model and this will be discussed in the following sections.

8.6. Implications toxicity of salinity and toxic relationships of mixtures of pesticides with salinity with water quality guidelines

Water quality guidelines for salinity, based only on direct toxic effects, are unlikely provide the intended level of protection, since the indirect effects of salinity have not been considered. As indicated in Table 8.3 the protective concentrations of salinity should be revised based on the information of trophic relationships of the taxa under

consideration. The application of appropriate safety factors will also be important in this exercise, since the taxa which do not have sufficient information can be protected with the safety factors.

There are no significant differences in salinity acclimation on the toxicities of individual chemicals studied (chronic and acute). However the trigger values based on the toxicities of single species would under protect the species in higher tropic levels since there can be cascading effects as a result of the adverse effects on the lower trophic levels. *P. subcapitata* was more sensitive to two herbicides than *D. carinata*. Therefore, in addition to direct toxicity effects there can be effects on the cladoceran due to limitations of algal food. Thus, trophic relationships are very important considerations in the protection of species.

The toxicities of mixtures showed different relationships for both species and there were changes in sensitivities of salinity-acclimatised cultures, especially in *P. subcapitata*. Additivity seems to be the most prevalent toxicity relationship when both species are considered (Tables 8.6 and 8.7). Therefore, incorporation of additivity in WQGs would provide substantial protection for the species. The Australian and New Zealand WQGs (ANZECC and ARMCANZ 2000) have recognized additivity in their implementation. Herbicide mixtures have recently been incorporated in the WQGs of Quebec, Canada (Ministère de l'environment du Québec 2001) and a mechanism to include herbicide mixture toxicity has been proposed for the Swiss water-quality guidelines (Chevre et al. 2006). However, the toxicity of mixtures that are synergistic is still not sufficiently addressed by these WQGs. High salinity acclimation (6000 μS/cm) caused an increase in synergism especially in *P. subcapitata* (a 7.3% increase). It will be worthwhile to consider this in designing safety factors in SSD and ecological risk assessment and therefore enable it to be incorporated into WQGs. The safety factors used to derive WQGs are designed to be applied to toxicity data for singles chemicals, not mixtures. Therefore, a different approach is required. An appropriate approach was developed by Ross and Warne (1997). They used probabilistic risk assessment methods to determine how toxic any selected percentage of mixtures were, compared to concentration addition. Thus they found that 50% of 973 mixtures had toxicity that conformed to antagonism and additivity, and that 95%

of mixtures had toxicities less than 1.5 times greater than concentration addition. The use of the above approach has the advantage of providing a flexible risk-based approach to setting WQGs. The exact level of mixture toxicity used can then be made as a policy decision.

A summary of the present study is presented in the Figure 8.2.

Pseudokirchneriella subcapitata, Daphnia carinata

Multigenerational exposure of salinity

P. subcapitata	*D. carinata*
Life history affected ≥ 3000 µS/cm. -growth rate -cell division rate -generation time -cell yield	Life history affected ≥ 5500 µS/cm. -mean total reproduction -adult body lengths -intrinsic rate of natural increase -ingestion rates

Acute and chronic toxicities (non-acclimatised)

	P. subcapitata (IC50)	*D. carinata (EC50)*
Salinity	5620 µS/cm	8813 µS/cm
Pesticides (moles/L)	Atr-2.22 X 10^{-7} Mol.-1.60 X 10^{-6} Chl.-2.27 X 10^{-6}	Atr-1.97 X 10^{-4} Mol.- 1.34 X 10^{-4} Chl.- 6.07 X 10^{-10}
Mixtures	Antagonism- 50% Additivity- 47% Synergism- 2%	Antagonism- 42% Additivity-47% Synergism-11%

Acute and chronic toxicities (salinity acclimatised)

	P. subcapitata (IC50)	*D. carinata (EC50)*
Salinity	5201µS/cm	9309 µS/cm
Pesticides	Atr-1.98 X 10^{-7} Mol.-1.51X 10^{-6} Chl.- 3.19 X 10^{-6}	Atr-1.92 X 10^{-4} Mol.- 1.47 X 0^{-4} Chl.- 5.25 X 10^{-10}
Mixtures	Antagonism-80% Additivity-20% Synergism-9%	Antagonism-37% Additivity-52% Synergism-11%

Summary

-The direct effects of both salinity and pesticides on *P. subcapitata* may have indirect effects on *D. carinata*. and should be considered in SSDs. Therefore, it is recommended that considering trophic relationships and community level effects in WQGs are considered.

- *D. carinata* that were acclimated to high salinity acquired some tolerance to acute toxicity of salinity. High salinity acclimated *P. subcapitata* did not develop any tolerance to salinity.

-High salinity acclimation of *D. carinata* did not change their sensitivity to pesticides. However, the sensitivity of *P. subcapitata* to pesticides was altered as a result of high salinity acclimation, as indicated by changes in occurrence of toxicity relationships i.e. additivity, antagonism and synergism.

-The ACRs of salinity for the two test species generated ranged from 0.8 to 2 and will be useful in determining appropriate safety factors for salinity.

-WQGs that incorporated mixture toxicity based on CA with properly derived safety factors (to minimise over protection and under protection) would be adequate to provide the expected level of protection for the species.

- Using the concentration addition (CA) model for the two test species provided adequate protection for approximately 90% of the mixtures tested.

Figure 8.2: Summary of the key findings of the project.

8.7. Summary and conclusions

The exposure to salinity caused long-term multigenerational and short-term acute toxic effects on the test species, *P. subcapitata* and *D. carinata*.

The direct acute effects of salinity on cladocerans occurred at $\geq$ 8800 µS/cm and chronic toxic effects on alga occurred at $\geq$ 5600 µS/cm. Cladocerans were significantly ($p \leq 0.05$) affected in terms of mean total reproduction, adult body lengths, intrinsic rate of natural increase and ingestion rates at salinities of $\geq$ 5500 µS/cm. Algae were significantly ($p \leq 0.05$) affected in terms of growth rate, cell division rate, generation time and cell yield at the salinity levels of $\geq$ 3000 µS/cm.

P. subcapitata did not develop salinity tolerance when acclimatised. In fact in one of the six experiments it became significantly less tolerant (at $p \leq 0.05$) while the other five were not significantly different. Being a good osmoregulator, acclimatised *D. carinata* became less sensitive to the acute toxicity of salinity. However, there was no evidence of acquiring salinity tolerance by both species in terms of the life-history traits studied in the multigenerational experiments.

Data generated showed that the alga, *P. subcapitata* was more sensitive to some toxicants compared with the cladoceran, *D. carinata*. Therefore, *D. carinata* may experience indirect effects at lower concentrations than those indicated by toxicity tests using *D. carinata*. If similar effects occur for species where *D. carinata* is the food source for them, shifting of the established species sensitivity distributions would occur. Therefore, the expected level of species protection may not be achieved using single species toxicity data. A method was developed for incorporating indirect toxicant effects. This yielded lower protective concentrations.

Acclimatisation of both test species (over multiple generations) to elevated salinity did not change their sensitivity to the three pesticides. For the cultures of both test species not acclimatised to elevated salinity the percentage of mixtures conforming to antagonism and additivity were similar (i.e., 40 – 50%), and only a small percentage

conformed to synergism. For both species, acclimatisation to elevated salinity led to an increase in sensitivity to the mixtures with increases in the percentages of mixtures that conformed to additivity and synergism and decreases in those that conformed to antagonism.

The present study clearly indicated that environmental conditions (i.e., salinity) influence the life history of organisms and also the toxic relationships of different toxicant mixtures. Therefore the effects of environmental variables such as salinity, pH (low pH often caused by acid sulphate soils) and temperature (due to seasonal variations in temperate countries) on toxic effects need to be studied further, since they affect the solubility, bioavailability transformation and degradation of toxicants in the environment.

It is important to supplement the WQGs based on single toxicant and single species data with mixture toxicity data (with realistic mixtures) and taking the community level effects (trophic relationships) of toxicants in revising WQGs. Therefore the promotion of research on such areas is recommended.

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